UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTY OF MEDICINE

DEPARTMENT OF MEDICINE

DOCTORATE THESIS

Role of the SET/PP2A signaling axis and its microRNA-mediated deregulation in colorectal cancer development: Potential clinical and therapeutic impact

Thesis submitted in fulfillment of the requirements for the Degree of International Doctor by Cristina Caramés Sánchez

Directed by Dr. Ion Cristobal and Dr. Jesús García-Foncillas

Presented as a publication compendium

Madrid, 2017

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE MEDICINA

DEPARTAMENTO DE MEDICINA

TESIS DOCTORAL

Papel del eje de señalización SET/PP2A y su desregulación mediada por microRNAs en el desarrollo del cáncer colorectal: Potencial impacto clínico y terapéutico

Memoria presentada para la obtención del grado de Doctor con mención internacional en Medicina por Cristina Caramés

Dirigida por el Dr.Ion Cristobal y Dr. Jesús García-Foncillas

Tesis presentada como compendio de publicaciones

Madrid, 2017

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Que Cristina Caramés Sánchez, ha realizado bajo nuestra dirección, el trabajo titulado "Role of the SET/PP2A signaling axis and its microRNA-mediated deregulation in colorectal cancer development: Potential clinical and therapeutic impact"

para optar al grado de Doctor Internacional en Medicina.

Dicho trabajo reúne, a nuestro juicio, las condiciones de originalidad y rigor necesarios, y por tanto, avalamos su presentación para ser juzgado

Para que conste, firmamos el presente certificado en:

Madrid, a 27 de Marzo de año 2017

Dr. Ion Cristobal Yoldi

Prof. Jesús García-Foncillas López

AGRADECIMIENTOS

Ya han pasado 7 años desde el momento en que decidí que quería formarme en la Fundación Jímenez Díaz y puedo afirmar con rotundidad que mis expectativas en “La Concha” han sido superadas con creces.

Por eso quiero aprovechar esta ocasión para agradecer el ingente trabajo de todas la personas que están liderando y trabajando por este gran proyecto asistencial, docente e investigador.

A la Dirección del Hospital, que ha incorporado a excelentes profesionales que han enriquecido mi servicio y otros muchos del hospital. Que nos ha motivado a salir al extranjero y conocer otros servicios excelentes proporcionándonos todo tipo de facilidades. Que cada año redobla esfuerzos para poder mejorar la oferta docente y asistencial y que ha impulsado el crecimiento del Instituto de Investigación Sanitaria para con todo ello mejorar la asistencia a los pacientes.

Agradecer a todos mis compañeros de trabajo todo el tiempo que han dedicado a enseñarme no solo como llegar a los diagnósticos mas certeros o los tratamientos más adecuados sino también lo importante que es proporcionar un trato exquisito a los pacientes informándoles de sus alternativas terapéuticas y respetando sus decisiones.

A la Unidad Docente, que ha puesto todo su empeño no solo en que mejorásemos nuestras competencias profesionales sino también en como debemos transmitírselas a los demás para que la formación sea una parte siempre presente en nuestro desarrollo profesional.

Al Dr. García-Foncillas por hacer realidad la Translational Oncology Division y el Oncohealth Institute que me ha permitido vivir la oncología en una situación privilegiada, al Dr. Ion Cristóbal por la co-dirección de esta tesis, al Dr. Rodés (DEP) por iniciarme y motivarme en la investigación clínica desde que empecé a estudiar primero de medicina y al Dr.Caramés (mi padre) por enseñarme la vocación de ser médico y desde que supo que quería dedicarme a esto, mostrarme “el arte” de la medicina con sus frustraciones y satisfacciones. Con el aprendí el significado del conocido aforismo de Hipócrates, “ Ars longa, vita brevis, occasio praeceps, experimentum periculosum, iudicium difficile”.

Y por último a mi querida familia y amigos que siempre me apoyan.

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STRUCTURE

This international mentioned doctorate thesis is presented as a compendium of the following publications that aimed to respond the five main objectives of the research work:

Article 1:

Cristobal I, Manso R, Rincon R, **Caramés C**, Senin C, Borrero A, Martínez Useros J, Rodriguez M, Zazo S, Aguilera 0, Madoz J, Rojo F, García-Foncillas J. PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. Mol Cancer Ther 2014;13(4):938-47.[1]

Article 2:

Cristóbal I, Manso R, Rincón R, **Caramés C,** Zazo S, del Pulgar T, Cebrian A, Madoz J, Rojo F and García-Foncillas J. Phosphorylated protein phosphatase 2A determines poor outcome in patients with metastatic colorectal cancer. Br J Cancer 2014;111(4):756–62.[2]

Article 3:

Cristóbal I,Rincón R, Manso R, Madoz J**, Caramés C,** del Puerto L, Rojo F and García-Foncillas J. Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation. Biochim Biophys Acta 2014;1842(9):1823–9.[3]

Article 4:

Cristobal I, Rincon R, Manso R, **Caramés C**, Zazo S, Madoz J, Rojo F, García-Foncillas J. Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer. Clin Cancer Res 2015;21(2):347–56.[4]

Article 5:

Cristóbal I *, **Caramés C *,** Rincón R, Manso R, Madoz J, Torrejón B, González-Alonso P, Rojo F, García-Foncillas J. Downregulation of microRNA-199b predicts unfavorable prognosis and emerges as a novel therapeutic target which contributes to PP2A inhibition in metastatic colorectal cancer. Oncotarget 2016;In press.[5]

*** These authors contributed equally to this work**

Article 6:

Caramés C, Cristobal I, Moreno V, Marín P, González-Alonso P, Torrejón B, Minguez P, Leon A, Martín J.I, Hernández R, Pedregal M, Martín M.J, Cortés D, García-Olmo D, Fernández M.J, Rojo F and García-Foncillas J. MicroRNA-31 Emerges as a Predictive Biomarker of Pathological Response and Outcome in Locally Advanced Rectal Cancer. Int J Mol Sci 2016;17(6):pii:E878.[6]

Article 7:

Caramés C, Cristóbal I, Moreno V, Moreno I, Rodriguez M, Marín JP, Correa AV, Hernández R, Zenzola V, Hernández T, León A, Martín JI, Sánchez-Fayos P, García-Olmo D, Rojo F, Goel A, Fernández M.J, García-Foncillas J. MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. Int J Colorectal Dis 2015;30(7):899-906.[7]

ABBREVIATIONS

5-FU: 5- Fluorouracil

AML: Acute myeloid leukemia

Anti-EGFR: Anti-epithelial growth factor receptor

Anti-VEGFR: Anti-vascular endothelial growth factor receptor

APC gene: Adenomatous polyposis coli gene

B-CLL: B-cell chronic lymphocytic leukemia

Bp: Base pair

CI: Confidence interval

CLL: Chronic lymphocytic leukemia

CML: Chronic myeloid leukemia

CRC: Colorectal cancer

CRT: Chemoradiotherapy

CT: Computed tomography

EGFR: Epithelial growth factor receptor

EMT: Epithelial mesenchyme transition

ESMO: European society medical oncology

FF: Fresh frozen

FFPE: Formalin-fixed paraffin-embedded

FN: False negative value

FOLFIRI: Folinic acid, irinotecan, fluoracil

FOLFIRINOX: Folinic acid, irinotecan, fluoracil, oxalipaltin

FOLFOX: Folinic acid, oxaliplatin, fluoracil

FP: False positive value

HCC: Hepatocelular carcinoma

IHC: Immunohistochemistry

LARC: Locally advanced rectal cancer

LAT: Local ablative therapy

LOHP: Oxaliplatin

mCRC: Metastatic colorectal cancer

MiR-21: MicroRNA-21

MiR-31: MicroRNA-31

MiR(s): MicroRNA(s) or MiRNA(s)

MMR: Mismatch repair

MRI: Magnetic resonance imaging

mRNA: Mesenger RNA

MSI-H: High levels of microsatellite instability

MSI: Microsatellite instability

NED: No evidence of disease

NGS: Next generating sequencing

NHL: Non-hodking lymphoma

Nt: Nucleotide

OS: Overall survival

p-PP2A-C Y307: Phosphorylated protein phosphatase 2A at tyrosine 307

p-PP2A: Phosphorylated protein phosphatase 2A

PCR: Polymerase chain reaction

PET-TAC: Positron emission tomography scan

PFS: Progression free survival

PP2A: Protein phosphatase 2A

PPP2R2A: Protein phosphatase 2A regulatory subunit B alpha isoform

PPP2R5E: Protein Phosphatase 2 Regulatory Subunit B'Epsilon

RDT: Radiotherapy

REMARK: Reporting Recommendtions for Tumor Marker Prognostic Studies

RNA: Deoxyribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

SCNAs: Somatic copy number alterations

SiRNA: Small interfering RNA

SNPs: Single nucleotide polymorphisms

TGF-α: Transforming growth factor-α

TMA: Tissue microarray

TME: Total mesorectal excision

TN: True negative value

TNM: Tumor node metastasis

TP: True positive

UTR: Untranslated region

SUMMARY (ABSTRACT)

Introduction

Colorectal cancer (CRC) is a common and lethal disease. One of the fundamental processes driving the initiation and progression of CRC is the accumulation of a variety of genetic and epigenetic changes in colonic epithelial cells. Over the past decade, major advances have been made in our understanding of cancer genetics and epigenetics, including microRNA (miRNA or miR) deregulation. As with gene mutations in the cancer genome, a subset of these epigenetic alterations, affecting miRs, are presumed to have a functional role in CRC. Those improvements in our knowledge of CRC pathogenesis have led to these alterations being developed as clinical biomarkers for prognostic and therapeutic applications. Progress in this field suggests that a better comprehension of CRC molecular landscape will be commonly used in the near future to direct the management of CRC. Despite this great progress, further molecular and clinical research is needed to better select patients for current and novel emerging therapies and also to more specifically design future clinical trials based on alternative molecular target therapies under investigation. Protein phosphatase 2A (PP2A) is a tumor suppressor consisting in a heterotrimeric complex that regulates many signaling pathways crucial for cancer cell transformation. Several distinct PP2A inhibitory mechanisms had been identified in tumor cells such as phosphorylation and/or deregulation of some PP2A subunits, miRs deregulation and overexpression of endogenous PP2A inhibitors. Those alterations had been reported in many cancer types as contributing mechanisms of malignancy and conferring a poor prognosis to some tumors. However, the importance and potential clinical value of those alterations as biomarkers for CRC as well as their role in the pathogenesis of metastatic CRC (mCRC) remained mostly unexplored. Moreover, miR-21 and miR-31 are miRs with an oncogenic function that had been reported as potential regulators of PP2A activity. Their deregulation has been described to play an important role in CRC development and they have been associated with 5- Fluorouracil (5-FU) resistance. However, the role of these miRs as predictive biomarkers of response to 5-FU based neoadjuvant chemoradiotherapy (CRT) in locally advanced rectal cancer (LARC) remained unexplored. In this PhD research, we investigated the role of PP2A and its regulatory mechanisms in CRC pathogenesis. Furthermore, we analyzed the potential impact of PP2A inhibition based biomarkers in mCRC and LARC.

Material and methods

In vitro studies: We used 9 types of human CRC cell lines and we performed several in vitro studies such as proliferation and cell viability assays, phosphatase activity assays, analysis of caspase activation, transfection experiments, colony-forming assays, western blot analysis and luciferase assays. Patient samples: To validate our in vitro results and to explore the potential clinical value of our proposed biomarkers, the study comprised fresh-frozen samples and formalin-fixed paraffin-embedded (FFPE) tumor samples of 522 CRC patients divided in 4 independent CRC patient cohorts that are explained in more detail later. To measure miR expression levels in tumor samples we performed real-time RT-PCR and SET, PP2A and p-PP2A was measured by both RT-PCR and immunohistochemistry (IHC). All the manuscripts elaboration was carried out in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines.

Results

In the first paper, we show that PP2A is frequently inactivated in patients with CRC. Moreover, we identified overexpression of the endogenous PP2A inhibitors SET and CIP2A and downregulation of the regulatory PP2A subunits PPP2R2A and PPP2R5E, as contributing mechanisms to PP2A inhibition in CRC. Thus, we further studied the relevance of each of those PP2A inhibitory mechanisms in CRC. In the second paper, we reported that phosphorylated protein phosphatase 2A at tyrosine 307 (p-PP2A-C Y307) is a molecular mechanism that contributes to PP2A inhibition in CRC and confers a worse outcome. This alteration was found in 17.2% of mCRC and the subgroup of high p-PP2A levels patients showed substantially worse overall survival (OS) (median 0S, 6.0 vs. 26.2 months, P< 0.001) and progression-free survival (PFS) (median PFS, 3.8 vs. 13.3 P< 0.001). Importantly, the multivariable analysis demonstrated that p-PP2A is an unfavorable independent factor associated with OS (hazard ratio 2.7; 95% confidence interval, 1.8 – 4.1; P< 0.001) and PFS (hazard ratio 3.0; 95% confidence interval; 1.8-5.0; P<0.001). In the third paper, we also reported that the restoration of p-PP2A using PP2A activating drugs such as forskolin is a potential alternative therapy with benefit in patients with high p-PP2A. In the fourth paper, we showed that SET overexpression promotes cell growth and colonosphere formation. Furthermore, we published that SET inhibits PP2A then impairing its antitumor effects and SET reduces sensitivity to oxaliplatin in CRC cell lines. Moreover, SET overexpression was detected in 24.8% of mCRC patients, determined significantly shorter OS (8.6 vs. 27 months, P< 0.001), PFS (7.1 vs. 13.7 months; P< 0.001) and poor response to oxaliplatin-based chemotherapy. To continue, in the fifth paper we show that miR-199b is downregulated in 4 out of 5 SET overexpressing CRC cell lines and that miR-199b expression is inversely correlated with SET overexpression in CRC patients. Moreover, we published that miR-199b led to PP2A activation through a SET inhibition impairing cell viability and enhancing oxaliplatin sensitivity in CRC cells. MiR-199b was found downregulated in 25% of cases, and associated with lymph metastasis (P= 0.049), presence of synchronous metastasis at diagnosis (P= 0.026) and SET overexpression (P< 0.001). Furthermore, low miR-199b levels determined shorter OS (30 months vs. 9.7 months, P< 0.001), PFS (15.4 vs. 8.6, P= 0.003) and predicted clinical benefit to oxaliplatin treatment. Finally, in papers sixth and seventh we showed how the potential PP2A regulators, miR-21 and miR-31, when they are found overexpressed are able to predict pathological response and outcome to neoadjuvant 5-FU based CRT in LARC. MiR-21 was found overexpressed in 77.6 % of LARC cases, and significantly correlated with pathological response (P= 0.013). The odds ratio of having miR-21 overexpression and not getting a pathological respond to CRT resulted in 9.75 (confidence interval (CI) 2.24 to 42). Sensitivity, specificity, negative predictive values, and positive predictive value were 86.6, 60, 42.8, and 92%, respectively. Multivariate analysis confirmed the clinical significance of miR-21 determining preoperative CRT response. In the other hand, high miR-31 levels were found overexpressed in 34.2% of cases. Its overexpression significantly predicted poor pathological response (P= 0.018) and a worse OS since 78% of the patients with high miR-31 expression were alive at 6 years follow up compared with 96% of those with low miR-31 (P= 0.008).

Conclusions

Altogether we have identified, the tumor suppressor activity of PP2A is commonly inhibited in CRC. SET overexpression, p-PP2A-C Y307, miR-199b downregulation, and the downregulation of the PP2A regulatory subunits PPP2R5E and PPP2R2E as contributing mechanisms to this PP2A inhibition in CRC. In addition, high levels of p-PP2A-C Y307 is an alteration that determines poor outcome in mCRC and SET overexpression is a frequent event in mCRC that plays a potential oncogenic role associated with worse outcome and resistance

to oxaliplatin. In the same way, miR-199b is a tumor suppressor miR whose downregulation independently determines worse outcome in mCRC and resistance to oxaliplatin therapy. Finally, deregulation of the PP2A potential modulators, miR-21 and miR-31, have clinical impact determining pathological response and outcome in LARC.

RESUMEN

Introducción

El cáncer colorrectal es una enfermedad letal y común. Uno de los procesos fundamentales que conllevan al inicio y progresión del cáncer colorrectal es la acumulación de alteraciones genéticas y epigenéticas en las células de la mucosa colónica. En las últimas décadas, se ha progresado mucho en el conocimiento de la genética y epigenética del cáncer colorrectal, incluyendo el mejor conocimiento de las alteraciones producidas por los microRNAs. De igual forma que ocurre con las mutaciones en los genes, algunas alteraciones epigenéticas que afectan a los microRNAs, conocidas como "eventos conductores" parecen tener un papel funcional muy relevante en este tipo de cáncer. Este avance en el conocimiento de la patogénesis del cáncer colorrectal ha permitido desarrollar biomarcadores pronósticos, predictivos y con aplicaciones terapéuticas, basados en dichas alteraciones. De hecho, el desarrollo de este campo sugiere que un mejor conocimiento del panorama molecular será utilizado en el futuro próximo para personalizar el tratamiento del cáncer colorrectal. No obstante, a pesar de estos grandes avances, se necesitan más estudios de investigación tanto moleculares como clínicos para así, seleccionar mejor a los pacientes para recibir las terapias actuales, las terapias nuevas y también para diseñar de forma más específica los futuros ensayos clínicos basados en terapias moleculares dirigidas. La proteína fosfatasa 2A (PP2A) es una proteína con actividad serine-treonina fosfatasa supresora tumoral compuesta por un complejo heterotrimérico que regula varias vías de señalización importantes y cruciales para la trasformación tumoral. Se han descrito algunos mecanismos moleculares por los cuales PP2A está inhibida tales como la fosforilación de la subunidad catalítica, la desregulación de sus subunidades reguladoras, la inhibición por microRNAs o por inhibidores endógenos como SET o CIP2A. Dichas alteraciones han sido descritas en algunos tipos tumorales como factores que contribuyen a malignidad. No obstante, la importancia de esas alteraciones basadas en la inhibición de PP2A en cáncer colorrectal no era conocida antes del inicio de este trabajo de investigación. Además, el microRNA-21 y el microRNA-31 son dos oncomicroRNAs bien conocidos en cáncer colorrectal que se han descrito como potenciales reguladores de la actividad de PP2A. Se ha visto que su desregulación juega un papel muy importante en el desarrollo de cáncer colorrectal y se han asociado a resistencia a terapias basadas en 5-fluoracilo. Sin embargo, el papel de estos dos microRNAs para predecir la respuesta a la quimioradioterapia neoadyuvante basada en 5-fluoracilo en cáncer de recto localmente avanzado no era conocido. En este trabajo de investigación, nosotros exploramos el papel de PP2A y sus mecanismos reguladores en la patogénesis del cáncer colorrectal. Además, analizamos el potencial de las alteraciones moleculares que se basan en la inhibición de PP2A como biomarcadores clínicos en cáncer colorrectal metastásico y cáncer de recto localmente avanzado.

Material y métodos

Estudios *in vitro*: Utilizamos 9 líneas celulares de cáncer colorrectal humanas con las que llevamos a cabo varios estudios in vitro tales como ensayos de proliferación, de viabilidad, de actividad fosfatasa, apoptosis, experimentos de transfección, western blot, ensayos de

formación de colonias y ensayos de luciferasa. Muestras de pacientes: Con el fin de validar los estudios *in vitro* y de explorar su potencial clínico como biomarcadores, el estudio comprendió muestras tanto parafinadas como congeladas en fresco de 522 pacientes divididos en 4 cohortes independientes que se explican con detalle más adelante. Para medir la expresión de los microRNAs en las muestras tumorales hicimos análisis de PCR en tiempo real y SET, PP2A y p-PP2A fueron medidos tanto por PCR en tiempo real como por inmunohistoquímica. Todos los artículos fueron elaborados en concordancia con las guías REMARK ("Reporting Recommendations for Tumor Marker Prognostic Studies")

Resultados

En el primer artículo mostramos como PP2A está inactivado en pacientes con cáncer colorrectal. Además, identificamos mecanismos moleculares que contribuyen a la inactivación de PP2A como la sobreexpresión de SET y CIP2A y la baja expresión de las subunidades reguladoras de PP2A, PPP2R2A and PPP2R5E. Así pues, seguimos investigando la relevancia de estas alteraciones en cáncer colorrectal. En el segundo artículo, publicamos que la fosforilación de la tirosina-307 en la subunidad catalítica de PP2A (p-PP2A-C Y307) es un mecanismo molecular que contribuye a la inhibición de PP2A y que confiere mal pronóstico. Esta alteración presenta una prevalencia del 17.2% en los pacientes con cáncer colorrectal metastásico y el subgrupo que la presenta mostró un peor pronóstico ya que la mediana de supervivencia global y la supervivencia libre de enfermedad resultaron de 6.0 meses y 3.8 meses respectivamente versus 26.2 y 13.3, meses respectivamente, para los pacientes con p-PP2A-C bajo. Además el análisis multivariante demostró que p-PP2A es un factor desfavorable independiente asociado a supervivencia global y progresión libre de enfermedad (hazard ratio 2.7; intervalo de confianza 95% 1.8–4.1; P< 0.001 y hazard ratio 3.0; 95% intervalo de confianza; 1.8-5.0; P< 0.001, respectivamente. En el tercer artículo, mostramos también que el restablecimiento de p-PP2A mediante fármacos que activan la función de PP2A es una potencial alternativa terapéutica para los pacientes que muestran niveles de p-PP2A altos. En el cuarto artículo, publicamos que la sobreexpresión de SET promueve la proliferación celular y la formación de colonosferas. Además, demostramos que la sobreexpresión de SET inhibe a PP2A y que por lo tanto promueve los efectos tumorales propios de la inhibición de PP2A como supresor tumoral y que SET reduce la sensibilidad al oxaliplatino en líneas celulares de cáncer colorrectal. Además la sobreexpresión de SET, se detectó en el 24.8% de los pacientes con cáncer colorrectal metastásico determinando de manera significativa una supervivencia global más corta (8.6 meses vs. 27 meses) y supervivencia libre a la progresión (7.1 meses y 13.7 meses). Así mismo la sobreexpresión de SET se asoció a resistencia a las terapias con oxaliplatino. Para continuar, en el quinto artículo, determinamos que la baja expresión del microRNA-199b, se encuentra en 4 de 5 líneas celulares con sobreexpresión de SET y que la expresión de SET y el microRNA-199b esta inversamente correlacionada. Además, la sobreexpresión de este microRNA conlleva a la activación de PP2A mediante la inhibición de SET y como consecuencia detiene la proliferación celular y aumenta la respuesta al oxaliplatino en líneas de cáncer colorrectal. El microRNA-199b se encontró con baja expresión en el 25% de los casos y se asoció de manera significativa al desarrollo de metástasis linfáticas, (P= 0.049), presencia de metástasis sincrónicas al diagnóstico (P= 0.026) y sobreexpresión de SET (P< 0.001). Así mismo la baja expresión del microRNA-199b determinó una supervivencia global más corta (30 meses vs. 9.7 meses, P< 0.001), una progresión libre de enfermedad más corta (15.4 meses vs. 8.6 meses, P= 0.003) y resistencia al tratamiento con oxaliplatino. Por último, en los artículos 6 y 7 mostramos el potencial de dos microRNAs que potencialmente regulan la actividad de PP2A, los microRNAs 21 y 31 como microRNAs con función oncogénica que son capaces de predecir la respuesta a la quimioradioterapia neoadyuvante en cáncer de recto. Así pues el microRNA-21 se encontró sobreexpresado en el 77.6% de los pacientes con cáncer de recto localmente avanzado y se correlacionó de manera significativa con la respuesta

patológica siendo el odds ratio de tener alto el microRNA 21 y no tener una respuesta patológica completa de 9.75 con un intervalo de confianza de 2.24 a 42. La sensibilidad, especificad, valor predictivo negativo y valor predictivo positivo fueron 86.6, 60, 42.8 y 92%, respectivamente. Además, el análisis multivariante confirmó la significación clínica del microRNA-21 determinando la respuesta a la quimioradioterapia neoadyuvante. Por otro lado, los niveles altos del microRNA-31 se encontraron sobreexpresados en el 34.2% de los casos y su sobreexpresión predijo de forma significativa una pobre respuesta patológica (P= 0.018) y una peor supervivencia global ya que el 78% de los pacientes con sobreexpresión del microRNA-31 estaban vivos a los 6 años de seguimiento frente al 96% de los pacientes que estaban vivos a los 6 años y tenían baja expresión del microRNA-31 (P= 0.008).

Conclusiones

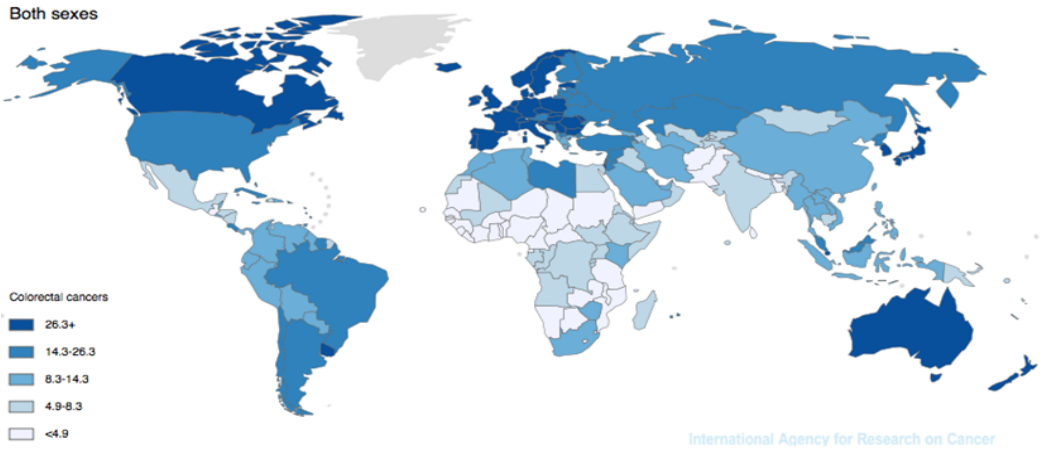
Con todo lo anterior, hemos identificado que la inhibición de PP2A es una alteración común en cáncer colorrectal y que está inhibición se produce por diferentes mecanismos moleculares como son: la sobreexpresión de SET, los niveles altos de p-PP2A-C Y307, la baja expresión del microRNA-199b y la baja expresión de las subunidades reguladoras de PP2A, PPP2R5E y PPP2R2E. Además, demostramos que los niveles altos de p-PP2A-C Y307, la sobreexpresión de SET y la baja expresión del microRNA-199b juegan un papel oncogénico y determinan un mal pronóstico en cáncer de colon metastásico asociando, en el caso de la sobreexpresión de SET y la baja expresión del microRNA 199b, resistencia a oxaliplatino. Por último, demostramos que la sobreexpresión de los microRNAs-21 y 31 tiene un impacto clínico ya que determina la respuesta a la quimioradioterapia neoadyuvante en cáncer de recto localmente avanzado.

INTRODUCTION

Colorectal cancer epidemiology, risk and preventive factors

CRC is a common and lethal disease. The risk of developing CRC is influenced by both environmental and genetic factors. Its incidence and mortality rates vary markedly around the world and globally CRC is the third most commonly diagnosed cancer in males and the second in females, with 1.4 million new cases and almost 694,000 deaths estimated to have occurred in 2012.[8] The highest incidence is reported in countries of Europe, North America, and Oceania, whereas incidence is lowest in some countries of Asia and Africa (Figure 1).

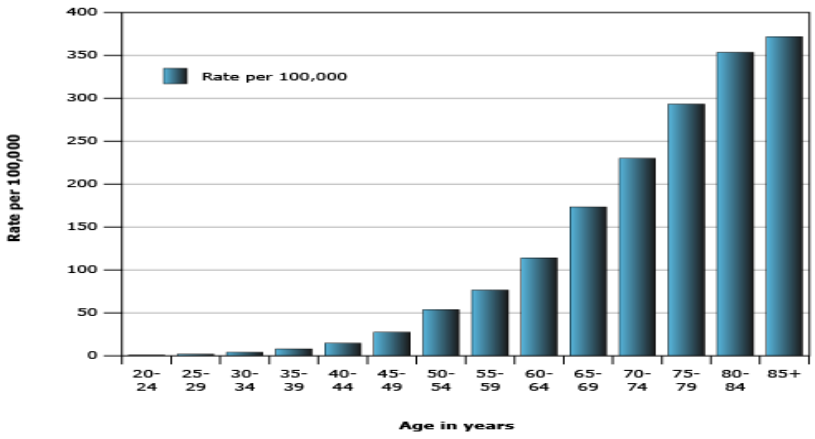
Figure 1. CRC cancer incidence



(Adapted from GLOBOCAN 2012)

However, rapid increases in previously low-risk countries, such as Spain and several countries in eastern Europe and east Asia, have been noted, which have been ascribed to changes in dietary patterns and risk factors towards a so-called western lifestyle.[9] In the other hand, in the USA and several other high-income countries, incidence has stabilized or started to decrease, probably because of increased use of early detection methods such as sigmoidoscopy and colonoscopy with polypectomy.[10] Global country-specific incidence and mortality rates are available in the World Health Organization, GLOBOCAN database. Incidence is low at ages younger than 50 years, but strongly increases with age (Figure 2).

Figure 2. CRC incidence by age



Median age at diagnosis is around 70 years in developed countries[11] and rates are higher in males than in females.

No single risk factor accounts for most cases of CRC. Apart from age and male sex, the following risk factors (which often co-occur and interact) have been identified and established in epidemiological studies: family history of CRC,[10] inflammatory bowel disease[12], smoking,[13] excessive alcohol consumption,[14] high consumption of red and processed meat,[15] obesity,[16] and diabetes.[17] Further emerging evidence suggests that infection with *Helicobacter pylori*, *Fusobacterium* spp, and other potential infectious agents might be associated with an increased risk of CRC.[18-19] Established preventive factors include physical activity,[20] use of hormone replacement therapy,[21] and aspirin,[22] with risk reduction in the order of 20–30%, and endoscopy with removal of precancerous lesions, for which the strongest risk reduction has been reported.[23] Although not as consistent, some data suggest a weak protective effect of diets rich in fruit, vegetables, cereal fiber and whole grains,[24] dairy products,[25] or fish[26] and, possibly, statin[27] therapy. Epidemiological studies[28] have consistently shown an inverse association between serum vitamin D concentrations and risk of CRC, but whether and to what extent this association is causal needs to be established (Table 1).

Table 1. CRC risk and preventive factors

Sociodemographic factors	**Risk**
Older age	Very strong increase
Male sex	Strong increase
Medical factors	
Family history	Strong increase
Inflammatory bowel disease	Strong increase
Diabetes	Moderate increase
Helicobacter pylori infection	Probable increase
Large bowel endoscopy	Strong reduction
Hormone replacement therapy	Moderate reduction
Aspirin	Moderate reduction
Statins	Probable reduction
Lifestyle factors	
Smoking	Moderate increase
Excessive alcohol consumption	Moderate increase
Obesity	Moderate increase
Physical activity	Moderate reduction
Diet factors	
High consumption of red and processed meat	Moderate increase
Fruit and vegetables	Probable reduction
Cereal fiber an whole grain	Probable reduction
Fish	Probable reduction
Dairy products	Probable reduction

CRC has also an important heritable component. Based on a large twin study,[10] 35% of CRC risk could be attributable to heritable factors. Apart from hereditary forms such as hereditary non-polyposis colon cancer (Lynch syndrome) and familial adenomatous polyposis, which are determined by well known genetic aberrations, but account for less than 5% of all CRC, genetic factors that determine the risk of disease are still incompletely understood.

Colorectal cancer molecular pathogenesis

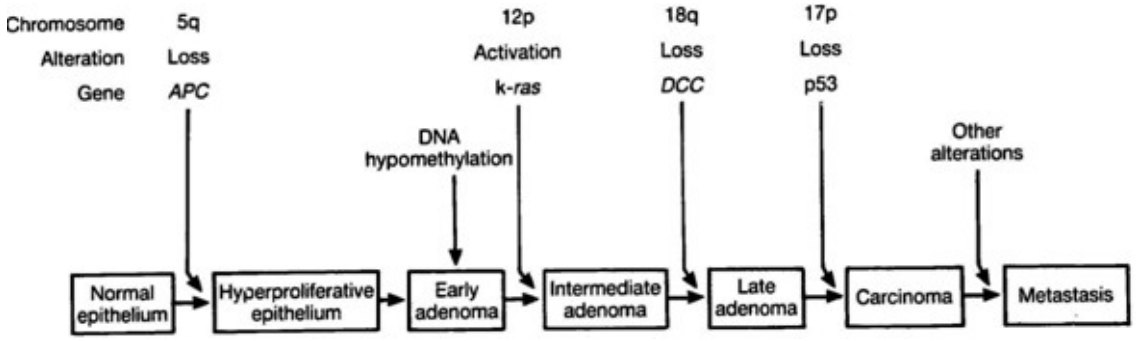
The molecular pathogenesis of CRC is heterogeneous and the molecular mechanisms underlying its development are clinically important because they are related to the prevention, prognosis and treatment response of the patient.[29] [30] Importantly, CRC is characterized by a progressive accumulation of genetic and epigenetic abnormalities in colon epithelial cells that lead to cancer progression.[31] [32] The level of understanding of the molecular events underlying CRC is far greater than for other common solid tumors. Specific germline mutations are responsible for the inherited CRC syndromes, while a stepwise accumulation of somatic mutations is thought to underlie most sporadic cases.

The adenoma –carcinoma sequence

The majority of CRCs are thought to arise from adenomas (adenomatous polyps) that become dysplastic. Adenomatous polyps form in the colon when normal mechanisms regulating epithelial renewal are disrupted. Surface cells lining the intestine are continuously lost into the bowel lumen due to apoptosis and exfoliation, and must be continuously replaced. Typically, proliferation occurs exclusively at the crypt base. As cells move towards the luminal surface, they cease proliferating and terminally differentiate. This ordered process is increasingly disrupted as adenomas increase in size, become dysplastic, and eventually attain invasive potential.

In 1990, Fearon and Vogelstein described the molecular basis for CRC as a multistep process in which each accumulated genetic event conferred a selective growth advantage to the colonic epithelial cell (Figure 3).[33]

Figure 3. A genetic model for colorectal tumorigenesis

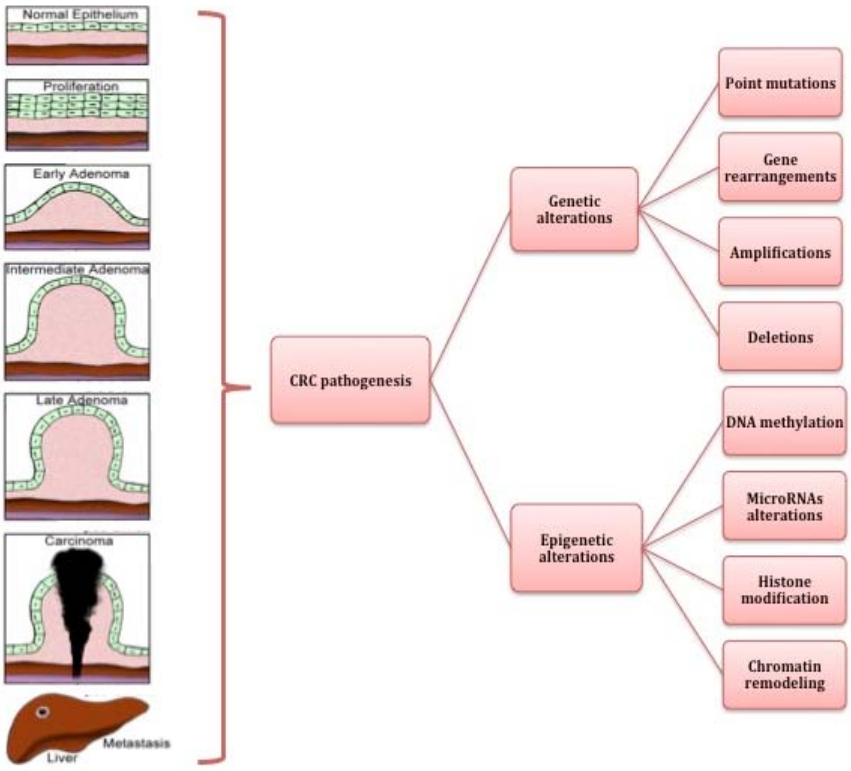


(Adapted from Fearon ER, Vogelstein. Cell 1990;61(5):759-67)

Later studies have served to further refine their hypothesis. According to the Vogelstein model, germline or somatic mutations are required for malignant transformation, and the accumulation of multiple genetic mutations rather than their sequence determines the biological behavior of the tumor. Germline mutations underlie the common inherited syndromes, while sporadic cancers result from the stepwise accumulation of multiple somatic

mutations. Mutations in the adenomatous polyposis coli (APC) gene, a feature common to both inherited and sporadic CRCs, occur early in the process, while p53 tumor suppressor gene mutations generally occur late. In addition to point mutations, other genetic changes are implicated in human tumorigenesis (gene rearrangements, amplifications, overexpression, and deletions).[32] Importantly, apart from those genetic alterations, CRC development is determined by de accumulation of epigenetics changes such as DNA methylation, miRs alterations, histone modification or chromatin remodeling. Those epigenetic alterations seem to be very import as early events and represent also driver events for CRC development (Figure 4).[31]

Figure 4. The adenoma carcinoma-sequence is determined by the accumulation of genetic and epigenetic alterations



Genetic and epigenetic alterations

The pathogenesis of CRC involves the accumulation of genetic and epigenetic modifications within pathways that regulate proliferation, apoptosis, and angiogenesis (Figure 4).

Genetic alterations

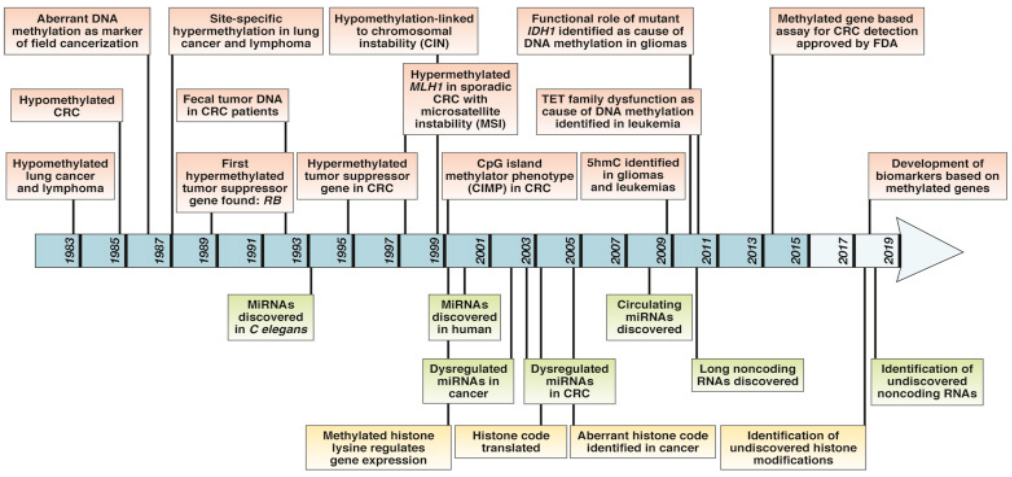
Sporadic CRCs arise from the accumulation of somatic genetic events that are clonal. These somatic defects may give a selective growth advantage to a cell, classifying the defect as a "driver" event and leading a cell clone to an increased proliferation that progress toward malignancy.[34] For sporadic CRC, it is estimated that among the 20,000 identified genes in the human genome, there are 138 driver genes (74 tumor suppressor genes and 64 oncogenes). A typical sporadic CRC, however, might only contain 2 to 8 driver gene alterations, with the remainder being "passenger" gene defects, as a result of genomic chaos and random events, and which have no effect on the neoplastic process.[35] This makes each CRC patient genetically

unique[36] and is an important item to consider for future approaches regarding personalized medicine. The median number of nonsynonymous mutations (that involves an amino-acidic change) in sporadic CRCs, regardless of pathogenesis, is 66 mutations per tumor, with only a handful of driver lesions and a larger number of passenger lesions. Integrated proteomic characterization of CRCs demonstrates the functional context for the observed mutations, with relatively few extending to the protein level.[37] An important concept is that a driver lesion for one patient's tumor might be different compared with another one. Common mutations, such as those seen with TP53 or KRAS, are dominant along the mutational landscape as driver mutations, but genes with relatively few mutations numerically outnumber the commonly targeted genes among CRCs. Because they are less commonly mutated overall, they are referred as the "tail" of the mutational frequency curve, but these genes are likely important as driver lesions among individual tumors.[38] They might also be considered individual or personalized biomarkers in the future.

Epigenetic alterations

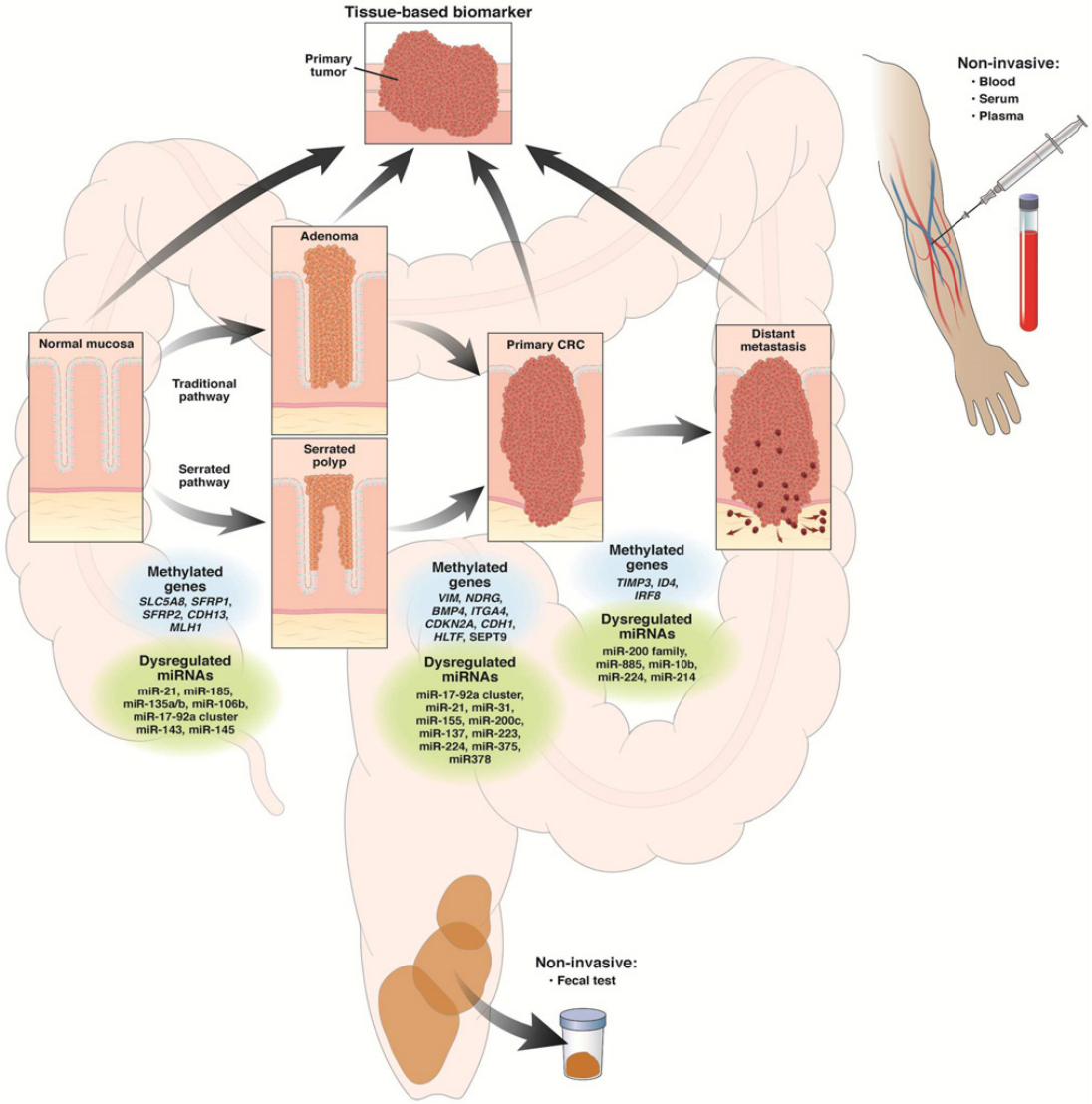
Epigenetic alterations have recently been recognized as significant contributors to cancer development. "Epigenetics" was first described by the developmental biologist Conrad H. Waddington in 1942 as "the study of heritable changes in gene expression mediated by mechanisms other than alterations in primary nucleotide sequence of a gene" and is now considered as broadly referring to heritable alterations in gene expression that are not mediated by changes in the DNA sequence. Epigenetic alterations frequently found in CRC cancer include aberrant DNA methylation, abnormal histone modifications, and altered expression levels of various noncoding RNAs, including miRs.[31] With regards to the role of epigenetic alterations in the normal-polyp-cancer sequence, as with gene mutations, it appears that a subset of the hundreds to thousands of alterations found in the typical cancer cell drives the initiation and progression of CRC formation through the sequential accumulation of genetic and epigenetic changes in key tumor-suppressors and oncogenes.[31] The prevailing consensus suggests that epigenetic alterations in CRC occur early and manifest more frequently than genetic alterations. In addition, advances in genomic technologies have led to the identification of a variety of specific epigenetic alterations as potential clinical biomarkers for CRC patients (Figures 5 and 6).

Figure 5: Discovery of various epigenetic alterations in CRC



(Adapted from Okugawa Y et al. Gastroenterology 2015;149 (5):1204-25)

Figure 6. Colorectal cancer epigenetics: from bench to bedside



(Adapted from Okugawa Y et al. Gastroenterology 2015;149(5):1204-25)

Colorectal cancer clinical management

Diagnosis

CRC may be suspected from one or more of symptoms and signs such as hematochezia, melena, abdominal pain, iron deficiency anemia, change in bowel habits, abdominal distention, nausea, vomiting, constipation etc. or may be asymptomatic and discovered by routine screening of average- and high-risk subjects. Once a CRC is suspected, the next test can be a colonoscopy or virtual colonoscopy. However, examination of tissue is required to establish the diagnosis and this is usually accomplished by colonoscopy.[10 39]

Staging

Once a CRC is detected a complete colonoscopy or a virtual colonoscopy is mandatory to detect synchronous cancers that are present in about 2–4% of patients. For rectal cancer, exact local staging at the time of diagnosis is essential and is the basis for requirement of

neoadjuvant treatment. Apart from the exact distance from the anal verge, definition of the local tumor extent is important. Endoscopic ultrasonography is accurate for determination of the T-stage of rectal cancer, and is the method of choice for regional tumors because of high accuracy to differentiate between non-invasive and invasive neoplasia. However, the most accurate method to define advanced T-stages is a magnetic resonance imaging (MRI).[10 40]

For both rectal and colon cancer, distant metastases should be ruled out because 20% of patients with newly diagnosed CRC have distant metastases at the time of diagnosis. The most common metastatic location is the liver followed by the lung. Thus, staging of CRC is generally advised to include a body computerized tomography scan (CT scan).[41] Although distant metastases can be identified in other organs including the bone and brain, no evidence supports routine investigation of these locations. Furthermore, data do not support routine use of positron emission tomography scan (PET-CT) in patients without suspected metastatic disease.[42]

CRCs are stage according to local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage) (Table 2). These stages are combined into an overall stage definition (Table 3), which provides the basis for therapeutic decisions.[36]

Table 2. Classification of CRCs according to local invasion depth (T stage), lymph node involvement (N stage) and presence of distant metastases (M stage)

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria*
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum¶
T4b	Tumor directly invades or is adherent to other organs or structures¶Δ
Regional lymph node (N)◊	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in one to three regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in two to three regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in four to six regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (eg, liver, lung, ovary, nonregional node)
M1b	Metastases in more than one organ/site or the peritoneum

(Adapted from Brenner et al. Lancet 2013; 383(9927):1490–502)

Table 3. TNM stage classification of CRC

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1 to T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3 to T4a	N1/N1c	M0
	T2 to T3	N2a	M0
	T1 to T2	N2b	M0
IIIC	T4a	N2a	M0
	T3 to T4a	N2b	M0
	T4b	N1 to N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

(Adapted from Brenner et al. Lancet 2013;383 (9927):1490–502)

Treatment

Like other patients with cancer, those with CRC should be assessing by a multidisciplinary team. The multidisciplinary team should include a colorectal surgeon, a medical oncologist, a gastroenterologist, a radiotherapist, a radiologist, and a pathologist. Depending on the tumor extent, the addition of a hepatic or thoracic surgeon is necessary. As mention before, the TNM staging classification provides the basis for therapeutic decisions.

Patients without distant metastases: Management of localized disease

Surgical resection

Approximately 80 percent of cancers are localized to the colon wall and/or regional nodes. Surgery is the only curative modality for localized colon cancer. The goal of surgery for invasive cancer is complete removal of the tumor, the major vascular pedicle, and the lymphatic drainage basin of the affected colonic segment. Thus, the treatment of choice for stage I, II and III colon cancer is oncological surgery resection.[43]

Adjuvant chemotherapy

For patients with CRC who have undergone curative resection of a colon cancer, the goal of postoperative (adjuvant) chemotherapy is to eradicate micrometastases, thereby reducing the likelihood of disease recurrence and increasing the cure rate.

Patients with stage III (node-positive) disease have a risk of recurrence ranging between 15% and 50%. The benefits of adjuvant chemotherapy have been most clearly demonstrated in

those patients with stage III, who have an approximately 30 percent reduction in the risk of disease recurrence and a 22 to 32 percent reduction in mortality with modern chemotherapy. Thus, adjuvant chemotherapy is recommended for all patients with stage III colon cancer without contraindications after curative resection. Regimens containing fluorouracil reduce recurrence rate by 17% units and increase OS by 13–15% units.[44] To improve disease-free survival and OS, several large prospective trials have investigated the addition of oxaliplatin to fluorouracil. The addition of oxaliplatin increased the absolute 5-year disease-free survival by 6.2 to 7.5% and the OS by 2.7 to 4.2% in patients with stage III colon cancer.[45 46] However, secondary subset analyses of two studies suggest that the benefit of oxaliplatin might be limited to patients younger than 65 years or younger than 70 years.[44] In large randomized trials, the addition of bevacizumab or cetuximab to an oxaliplatin- containing regimen did not show any benefit on disease-free survival. Additionally, the use of irinotecan combined with fluorouracil did not show any benefit and was associated with increased toxic effects.[47]

Stage II colon cancer is associated with statistically significant better disease-free survival and OS than stage III colon cancer. Among those patients, the benefits of chemotherapy are controversial, as is the relative benefit of an oxaliplatin as compared with a non-oxaliplatin-based regimen. The survival benefit from adjuvant chemotherapy seems to be reduced, and thus is generally recommended only for patients at high risk of relapse (T4 tumors, perforated tumors, bowel obstruction at the time of surgery, and <12 lymph nodes removed, poorly-differentiated histology; lymphovascular or perineural invasion). It is also important to evaluate the mismatch repair enzyme status, the assessment of comorbidities and anticipated life expectancy, and given the relatively good prognosis of stage II disease, the potential risks associated with treatment.[48]

Adjuvant chemotherapy carries a risk for significant toxicities, including mucositis, emesis, diarrhea, febrile neutropenia, fatigue, hair loss, hand-foot syndrome and cardiotoxicity. The frequency and severity of these side effects vary according to the specific drugs used and how they are administered. Fortunately, most of these symptoms are reversible with cessation of chemotherapy, and late and long-term effects are relatively infrequent with the exception of oxaliplatin-related peripheral neuropathy, which may persist.

Neoadjuvant therapy

This approach in CRC is limited to rectal cancer tumors. The treatment of choice for locally advanced rectal cancer (LARC) involves a multidisciplinary approach and consist in preoperative (neoadjuvant) chemoradiotherapy (CRT) followed by total mesorectal excision (TME) surgery.[49] The superiority of preoperative over postoperative CRT regarding local control, treatment compliance, and toxicity profile was demonstrated after CAO/ARO/AIO-94 (Working Group of Surgical Oncology/ Radiation Oncology/Medical Oncology of the German Cancer Society) trial. Several studies using 10-year follow-up outcomes have confirmed these findings.[50]

Patients with stage I rectal cancer should not be given any treatment in addition to surgery because the local recurrence rate is low (about 3%) and the benefit from neoadjuvant treatment very small.[51] Patients with stage III disease benefit from additional treatment, whereas the benefit for patients with stage II disease is less clear.[52 53] Currently, the neoadjuvant CRT regimen most widely used is based on fluoropyrimidines concurrent with conventional fractionation radiotherapy. Nevertheless, more than one-third of cases develop distant metastasis within 10 years from diagnosis and complete pathological response only occurs in 8% to 14% of patients. Thus, those patients who do not respond suffer undesired toxicities and delays in the resection of the primary tumor.

Patients with distant metastases: Management of metastatic disease

Approximately 20 percent of newly diagnosed colon cancers are metastatic at presentation (synchronous metastasis). This stage compromised by far the group of patients with a worse prognosis although, over the last 20 years, and the last decade in particular, the clinical outcome for patients with metastatic CRC has improved greatly due not only to an increase in the number of patients being referred for and undergoing surgical resection of their localized metastatic disease but also, to a more strategic approach to the delivery of systemic therapy and an expansion in the use of ablative techniques.

The role of the multidisciplinary team

The first step in the treatment of mCRC is for the multidisciplinary members to critically define whether or not a patient has initially clearly resectable or initially unresectable metastatic disease and to define the status of the resection of the primary tumor when considering the management of both synchronous and/or oligometastatic CRC. Conversely, for patients whose disease is deemed 'never to be resectable', the discussion may be left to the treating medical oncologist and patient as to the pros and contras of various approaches and sequences based on the perceived aims (duration of disease control versus quality of life, toxicity profiles, etc.)

The general condition and performance status of a patient are strong prognostic and predictive factors for chemotherapy. Whether a patient is classified as 'fit' or 'unfit' is now used to determine whether or not they will be assigned to a more intensive (combination of 2 or 3 cytotoxics with a biological) or less intensive treatment approach with the classical drivers of treatment choice being tumor, patient and treatment characteristics (Table 4).

Table 4. Revised ESMO groups for treatment stratification of patients according to whether patients are "fit" or "unfit

Patient´s classification	"Fit" patients		**"Unfit" patients**
	Group 1	Group 2	
Clinical presentation	A) Conversion and achievement of NED	Assymptomatic patients	Best supportive care
	B) Impeding clinical threat, organ dysfunction and severe symptons	No impeding clinical threat	
		Resection not an option	
Treatment goal	A) Cytoreduction followed by R0 resection, NED achieved by LAT	Disease control and hence prolonged survival	Palliative
	B) Improvement of symptons and hence avoidance of rapid evolution and prolonged survival		

LAT, local and ablative therapy; mt, mutant; NED, no evidence of disease; wt, wild-type

(Adapted from Van Cutsem et al. Annals of Oncology 2016;27(8):1386–1422)

Historically, 'fit' patients with mCRC were categorized according to the previous ESMO consensus guidelines into four groups (0, 1, 2, and 3) to determine the strategic treatment approaches (Table 5).[54 55]

As mentioned before, the decision as to whether a patient has initially resectable or initially unresectable metastatic disease should be made at the first meeting of the multidisciplinary team. Patients with initially resectable metastatic disease should be referred for immediate resection or perioperative chemotherapy with the goal being to achieve complete R0 resection and/or a situation where the patient can be treated with another ablative treatment.

However, in the case of fit patients with mCRC, whose metastases are not initially resectable, it is becoming increasingly obvious that the original ESMO groups 1 and 2 are becoming less clearly delineated and the treatment strategies less strict (Table 5).

Table 5. Historical ESMO groups for treatment stratification of fit patients with mCRC

	Group 0 Resecable	Group 1 Potencially resectable	Group 2 Not resectable	Group 3 Not resectable
Clinical presentation	Clearly resectable R0	Unresectable which might become resectable after response to conversion therapy	Multiples metastases/sites Tumor-related siymptons Able to withstand intensive therapy	Assymtomatic Multiple metastases Never able to undergo resection Unsuitable for intensive therapy Frail with co-morbidities
Treatment goal	Cure (NED)	Maximum tumor shrinkage	Clinically relevant tumor shrinkage Disease control	Slow tumor progression Tumor shrinkage less relevant Tolerability most relevant
Treatment intensity	Surgery	Intensive treatment approach; upfront most active combination rwgimwn	Intensive treatment approach; upfront most active combination rwgimwn	Less intensive treatmen approach (single agent or doublet with low toxicity)

(Adapted from Van Cutsem et al. Annals of Oncology 2016;27(8):1386–422)

Indeed, two clinically relevant categories are evolving for the systemic treatment of 'fit' patients with CRC whose metastatic disease is not resectable at presentation:

1) Those for whom intensive treatment is appropriate with the goal of cytoreduction (tumor shrinkage) and conversion to resectable disease (Esmo 1 group) or those who need intensive treatment, although they will never make it to resection or local ablative treatment, since they need a rapid reduction in tumor burden because of impending clinical threat, impending organ dysfunction or severe symptoms (Esmo 2 group).

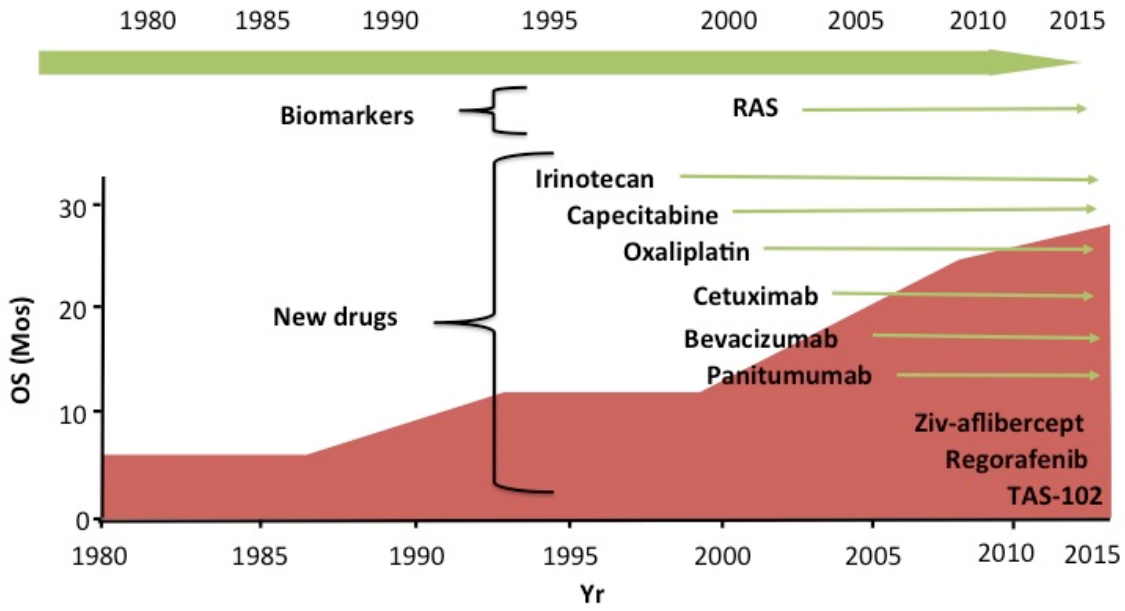
2) Those for whom intensive treatment is not necessary and where the goal is disease control (Esmo 3 group).

For patients in both categories, knowledge of the RAS and BRAF mutational status of their disease is used to further refine treatment strategies (Table 4).

Systemic therapy options for metastatic colorectal cancer

For decades, 5-FU was the sole active agent. This has changed markedly since the year 2000 to 2016, with the approval of irinotecan, oxaliplatin, bevacizumab, cetuximab, panitumumab, aflibercept, regorafenib and TAS-102 (Figure 7).[56] The best way to combine and sequence these agents is still not established.

Figure 7. Systemic therapy options for mCRC



First-line therapy

The typical first-line chemotherapy backbone comprises combination chemotherapy with a fluoropyrimidine plus oxaliplatin or irinotecan (FOLFOX or FOLFIRI). This provides higher response rate, better OS and progression-free and than a fluoropyrimidine alone. Infusional regimens of leucovorin/5-FU are generally less toxic than bolus regimens and in preference should be used. The oral fluoropyrimidine capecitabine can be used as an alternative to 5-FU/leucovorin alone [57] and in combination with oxaliplatin. Capecitabine is less frequently used in combination with irinotecan due to early concerns that it was more toxic than FOLFIRI.[58] However, the results are controversial.[59]

The monoclonal antibodies bevacizumab, an anti-vascular endothelial growth factor receptor (anti-VEGFR), and cetuximab and panitumumab, anti-epithelial growth factor receptor (anti-EGFR) have been shown to improve the clinical outcome of patients with mCRC when combined with combination chemotherapy regimens (FOLFOX, FOLFIRI or CAPOX) in the first-line setting.[60] [61] This combination of chemotherapy and monoclonal antibodies, if there are no contraindications, is the treatment of choice for patients with mCRC in the first line. The RAS mutational status determines whether a patient benefit or not of anti-EGFR therapies, because is a biomarker for therapy resistance. Thus, mCRC with RAS mutated are going to receive the combination chemotherapy with bevacizumab. For patients with RAS wild-type, currently, there is no evidence for the superiority of one class of biological over another (EGFR antibody therapies versus bevacizumab) in the first-line treatment. However, the combination

of chemotherapy with an EGFR antibody led to an improved response rate in both phase III trials (FIRE 3 study and CALGB) and to an improved OS in the FIRE 3 study, but not in the CALGB study. About PFS, it was identical for bevacizumab- and cetuximab-containing combinations in both phase III studies.[62 63]

The triplet combination chemotherapy regimen FOLFOXIRI has been demonstrated to be superior to FOLFIRI in selected patients.[64] FOLFOXIRI plus bevacizumab has also been shown to be superior to FOLFOX6 plus bevacizumab and FOLFIRI plus bevacizumab with more toxic effects.[65 66] However, the superiority of the cytotoxic triplet over a cytotoxic doublet has not been demonstrated in all studies[67] and the contribution of bevacizumab in the triplet combination is also uncertain. Thus, the therapy options for patients with mCRC in the first-line setting are for most patients a cytotoxic doublet such as FOLFOX, FOLFIRI or CAPOX and, in very selected patients the cytotoxic triplet FOLFOXIRI or fluoropyrimidine monotherapy (those monotherapy patients, in selected patients with asymptomatic primarily unresectable metastases that are likely to be eligible for multiple lines of treatment and who are not candidates for a combination chemotherapy).

Historically, continuing patients on chemotherapy until unacceptable toxicity or disease progression has been routine in clinical trials. However, clinical trials using this approach as well as clinical observations made during routine practice have indicated the dangers of continuing cytotoxic therapy, specifically oxaliplatin-containing therapy, as cumulative toxicity often occurs before clinical progression. As a result, discontinuation and/or intermittent combination chemotherapy/maintenance strategies have been investigated in a number of clinical trials with the result that these approaches provide an attractive treatment option for patients with a response or stable disease. In this context individualization and discussion with the patient is essential.

Second-line therapy

Second-line therapy describes the therapy received from the time that the first-line chemotherapy backbone has to be changed, mostly after failure of a first-line strategy. Second-line therapy is normally proposed for patients with good performance status and adequate organ function, and is dependent on the first-line therapy choice. Second-line therapy with oxaliplatin and irinotecan is known to be superior to best supportive care and 5-FU.

In patients in whom the initial chemotherapy backbone has failed, the chemotherapy backbone should be changed. Patients who receive FOLFIRI up front should receive FOLFOX and those patients who receive FOLFOX up front should receive an irinotecan-containing regimen, preferably FOLFIRI, with early evidence of the efficacy of this strategy provided by the trial of Tournigand et al.[68]

If bevacizumab was not used as the biological first line, it should be considered in second line, as FOLFOX plus bevacizumab was shown to improve OS compared with FOLFOX alone in a phase III trial and confirmed in subsequent studies.[69 70] Data from the randomized phase III TML study,[69] and from the BEBYP study,[71] showed continuation of bevacizumab treatment with second-line chemotherapy to benefit patients previously treated with bevacizumab, suggesting that patients treated first line with bevacizumab can benefit from subsequent therapies that target VEGF. The anti-angiogenic fusion protein aflibercept has been shown to confer a survival advantage when added to FOLFIRI in patients previously progressing on a prior oxaliplatin- containing regimen compared with FOLFIRI plus placebo.[72] A benefit has also been reported for patients treated with aflibercept who had received prior bevacizumab therapy.[73] Recently, a similar OS benefit has been reported for the anti-VEGFR2 antibody

ramucirumab, also in combination with FOLFIRI, as second-line treatment following first-line treatment with a fluoropyrimidine, oxaliplatin and bevacizumab.[73]

Both EGFR antibodies, cetuximab and panitumumab, have been shown to increase response rate and PFS, but not OS when combined with irinotecan-containing therapy in the second-line treatment setting[74] and can be considered if not used previously in the treatment of patients with RAS wild-type disease. However, generally, there is a similar relative benefit when cetuximab (and panitumumab) is used in later lines compared with second line.

No randomized phase III studies have been carried out which compare the different biologicals available, for patients that progress on a first-line bevacizumab-containing regimen.

Third-line therapy

Both cetuximab and panitumumab have shown efficacy in the third-line/salvage-therapy setting in patients with RAS wild- type tumors[75] [76] and are equally active as single agents.[77] The combination of cetuximab with irinotecan is more active than cetuximab alone, in irinotecan refractory patients. Any activity in patients with BRAF-mutant tumors seems to be limited to patients with chemorefractory mCRC.[78] There is no unequivocal evidence to support administration of the alternative EGFR antibody, if a patient is refractory to the other.

The multi-targeted kinase inhibitor regorafenib has reported activity versus placebo plus best supportive care in two phase III trials.[79] [80] Regorafenib has demonstrated a significant improvement in OS in patients pre-treated with all available cytotoxics and bevacizumab and EGFR antibodies, so it can be proposed as a standard treatment in this setting. However, some concerns over safety have raised some doubt as to whether the labeled dose (160 mg/day day 1–21 q4 weeks) is the optimal dose. In reality, it seems that in some regions many physicians start with a lower dose and then increase the dose to the approved dose if no toxicity is observed. Frequent and close monitoring for regorafenib toxicity is recommended.

Recently, an oral agent that combines trifluridine and tipiracil hydrochloride (TAS-102) has been shown to be effective in the treatment of patients with refractory mCRC, leading to a significant survival benefit that is similar to that of regorafenib, but with limited toxicity and is therefore a potential new option.[81]

Prognostic and predictive factors: the need of robust biomarkers to personalized colorectal cancer therapies

Biomarkers can be diagnostic, predictive or prognostic. Ideally, a biomarker should only serve one of these purposes, but there are good and clinically relevant examples of prognostic biomarkers that predict a response to a specific therapy, for example, human epidermal growth factor receptor 2 (HER2) in breast cancer [54] and BRAF (strongly prognostic and, to a lesser extent, predictive) in CRC.[82]

The best way to combine and sequence all the agents mentioned before for the treatment of mCRC is still not fully established. The introduction of biological therapy and chemotherapy combination has provided significant advances in survival as mentioned before. However, not all patients benefit from such therapies. Some patients do not obtain any benefit and suffer unnecessary side effects while others initially benefit but develop secondary resistance.[83] In this context, it is increasingly being realized that biomarkers for CCR management are necessary.

Thus, because only a subset of patients responds to each therapy and also to better define treatment strategies and follow up of patients with CRC, it is a priority to identify markers of prognosis and markers of response and resistance to therapy. Furthermore, considering the

high cost of these therapies and the management of CRC predictive and prognosis markers are not only a clinical necessity but are also an economic requirement.[84]

Currently, the biomarkers described for CRC are the following:

RAS mutational status

The cumulative data from clinical trials like the PRIME, OPUS, CRYSTAL, FIRE-3 and the phase III 20020408 trial, clearly show that patients whose tumors harbor any RAS mutation are unlikely to benefit from EGFR antibody therapy. The presence of a RAS mutation, according to expanded RAS analysis, which include at least KRAS exons 2, 3 and 4 (codons 12, 13, 59, 61, 117 and 146) and NRAS exons 2, 3 and 4 (codons 12, 13, 59, 61 and 117) is as a negative predictive marker of treatment outcome in patients with mCRC who might be under consideration for EGFR monoclonal antibody therapy.[54 62 85 86]

Thus, cetuximab and panitumumab should only be considered for the treatment of patients with RAS wild-type mCRC and expanded RAS analyses should be conducted on all patients being considered for EGFR antibody therapy.[54]

BRAF mutations status

BRAF, nearly always V600E mutations, are found in between 8% - 12% of mCRC included in clinical trials and are almost exclusively with RAS mutations.[87]

BRAF mutations are a significant negative prognostic marker for patients with mCRC. Median survival for patients with BRAF-mutant mCRC resulted in 10.4 months compared with 34.7 months for patients with BRAF wild-type tumors in a clinical trial reported by Tran et al. This poor prognosis for patients with BRAF-mutant tumors is confirmed by a number of randomized studies with specific chemotherapy regimens.[62 75 85 86]

The evidence of BRAF mutations as a negative predictive biomarker for EGFR antibody therapy in later lines is accumulating.[78 88] However, its role in earlier lines in combination studies with chemotherapy has not been ascertained.[75] Indeed, two meta-analyses[89 90] reported the efficacy benefit of EGFR antibody therapies to be greater in patients with RAS wild-type/BRAF wild-type tumors than in those with RAS wild-type/BRAF-mutant tumors. In the meta-analysis that included two trials involving chemorefractory patients and two second-line trials, the lack of a significant efficacy benefit by anti-EGFR treatments over standard chemotherapy alone in CRC BRAF-mutant tumors was considered to support the assessment of tumor BRAF mutation status before the initiation of anti-EGFR therapy. Conversely, researches of the second meta-analysis showed that there was not enough evidence to exclude anti-EGFR treatments from patients with RAS wild type and BRAF- mutant disease. Importantly, in a small analysis of the TRIBE study, the subgroup of CRC patients with BRAF-mutant tumors treated with the chemotherapy triplet FOLFOXIRI plus bevacizumab showed a non-statistically significant increase in OS compared with those treated with FOLFIRI plus bevacizumab.[91]

MSI status

In patients with localized CRCs, tumors that are MSI-H are associated with longer survival than either MSI-low or microsatellite-stable tumors.[92] In addition to the better prognosis afforded by the presence of MMR deficiency in an individual tumor, the bulk of available data support that adjuvant 5-FU based chemotherapy is less beneficial for patients with MSI-positive tumors.[93]

The prognostic influence of microsatellite instability is less clear in patients with mCRC, a population in which the prevalence of MSI-H disease is low (approximately 3.5 percent). At least one report suggests an adverse influence of microsatellite instability on prognosis in this setting, a finding attributed, at least in part, to the higher frequency of BRAF mutations in this population.[94]

Furthermore, emerging data have shown MMR status to predict the clinical benefit of immune check- point blockade with pembrolizumab in patients with mCRC. The immune-related objective response rate and immune-related 6-month PFS rate were 40% (4 out of 10 patients) and 78% (7 out of 9 patients), respectively, for patients with MMR deficiency CRC and 0% and 11% for those with MMR-proficient CRC, with excellent median PFS and survival (not reached) in the cohort with MMR deficiency CRCs versus 2.2 and 5.0 months, respectively, in the cohort with MMR-proficient tumors.[95]

Emerging biomarkers

Beyond MMR status, RAS and BRAF mutational status, a list of biomarkers status is emerging which may impact on the management of CRC and the response to all classes of targeted therapies. The independent influence of those biomarkers to use in clinical routine remains unproven. Variability in assay methodology, conflicting results from various studies examining the same factor, and the prevalence of multiple small studies that lack statistically robust, multivariate analyses all contribute to the lack of conclusive data. [96-97]

Protein phosphatase 2A (PP2A)

Relevance of phosphatases in cancer development

In normal cells, phosphoregulation is under the control of protein kinases and phosphatases. Thus, a correct balance between kinase and phosphatase activities is essential in maintaining cell homeostasis and to prevent cancer progression.[98]

Alterations affecting kinase activity have been shown to be recurrent in many cancers. Furthermore, treatments using kinase inhibitors have progressively been investigated in the last decade. However, the role of phosphatases remains in comparison underexplored. While aberrant global phosphorylation derived by the activation of protein kinases leads to aberrant activation of signaling pathways linked to neoplastic transformation, protein phosphatases invest a major role in negatively regulating such molecular networks. Indeed, the loss of function of different tumor suppressor phosphatases has been detected in many cancers.[36]

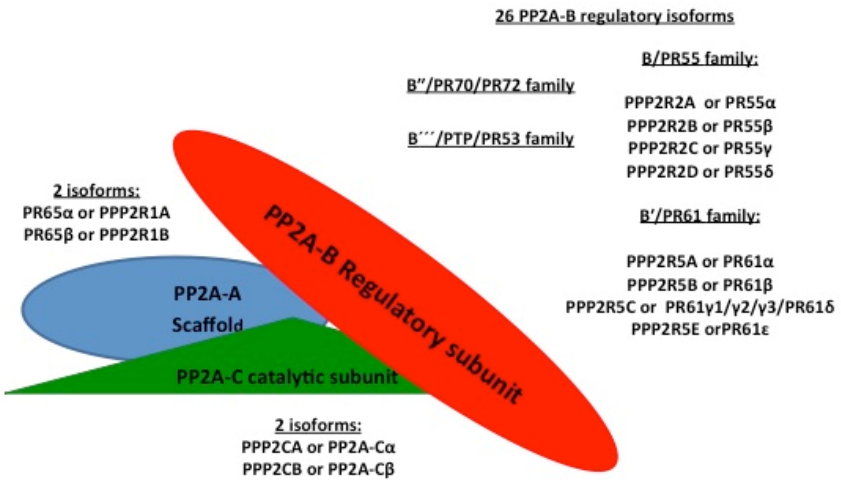
The first phosphatase described as such was PTEN, a dual-specificity phosphatase that has been found genetically or functionally inactivated in glioblastomas, prostate and breast cancer, endometrial neoplasms and hematological malignancies.[99] PTEN functions by inducing apoptosis and/or and inhibiting cell growth, adhesion and migration.[100]

PP2A structure

PP2A represents a major serine/threonine phosphatase that together with protein phosphatase 1, accounts for over 90% of all serine/threonine phosphatase activity in the cell.[101] PP2A is not a single entity but a heterotrimeric complex consisting of a scaffold PP2A-A subunit, a catalytic PP2A-C subunit and a regulatory PP2A-B subunit that determines both the specific substrate and the localization of the holoenzyme. Two isoforms have been described for PP2A-A and PP2A-C and at least 26 isoforms for PP2A-B which are classified within 4

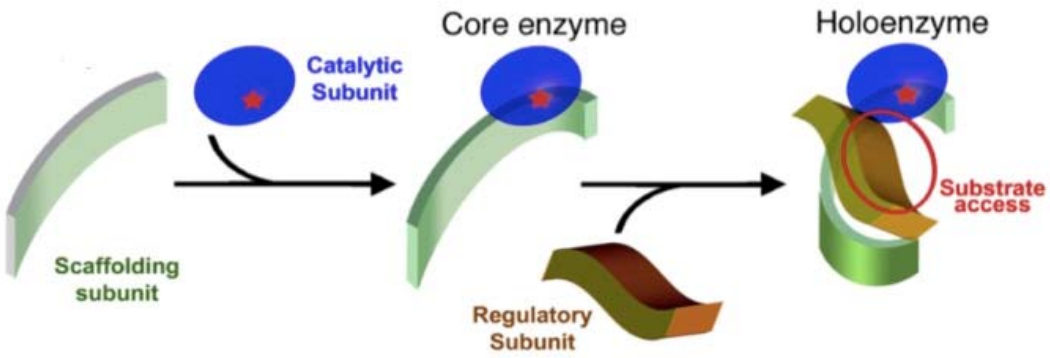
families. Therefore, PP2A can form a high number of different complexes that interact with many different substrates (Figure 8).

Figure 8. PP2A heterotrimeric complex



Typically, the catalytic subunit (PP2A-C) interacts with the structural or scaffold subunit (PP2A-A) making up the core of the enzyme. The association with a wide variety of B regulatory subunits to the core enzyme results in the formation of heterotrimeric PP2A holoenzyme complexes with diverse specificities and functions (Figure 9).

Figure 9. PP2A assembly



(Adapted from Xu Y et al, Cell 2006;127(6):1239–51)

The catalytic subunit (PP2A-C): It has a large conserved domain that forms a bimetallic active site for phosphorester hydrolysis. It targets phosphate groups on either serine or threonine residues and under some conditions harbors activity towards phosphorylated tyrosine. PP2A catalytic activity is encoded by two distinct ubiquitously expressed genes,[101] the Cα and the Cβ subunits. Both are 35 kDa in size and share 97% sequence identity. The levels of PP2A-C are tightly regulated in the cell at the translational or post- translational level. Loss of Cα in yeast and in mice is lethal[102] underscoring the importance of this phosphatase for proper

maintenance of cellular homeostasis.

PP2A-C phosphorylation appears to occur at either Tyr307 or Thr304[103] and as a consecuencue PP2A activity is reduced through several mechanisms. For example, phosphorylation of Tyr[307] inhibits the recruitment of the PPP2R2B and some others PP2A regulatory isoforms to the core enzyme.[104] Interestingly, this site is regulated by the oncogene c-SRC raising the possibility that inhibition of one or all of these subunits may be one of the requirements for c-SRC induced transformation.[101] Similarly, the C-terminal tail can be phosphorylated at Thr[304], which was also shown to inhibit the binding of PR55B family subunits but not the other B subunits.[101]

The structural subunit (PP2A-A): The catalytic subunit interacts with the PP2A-C scaffold protein at four C-terminal Huntington/elongation/A-subunit/TOR (HEAT) repeats.[105] Upon the formation of the core enzyme the scaffold protein folds in on itself forming a more horseshoe shape-like structure.[106] [107] The bending of the structural subunit allows the catalytic subunit unimpeded access to the PP2A substrate, which is recruited to the holoenzyme by the B regulatory subunit. Two alternative genes, PPP2R1A and PPP2R1B encode the two flavors of the PP2A-A scaffold protein [101]. Most PP2A holoenzymes contain the PPP2R1A isoform, while only a small fraction (10%) containing the PPP2R1B isoform. These two alternative transcripts differ in their ability to interact with the various regulatory B subunits.[108] Interestingly several tumor-specific mutations have been identified in PP2A-A which disrupt its ability to form holoenzymes with specific regulatory subunits.

The regulatory B subunits (PP2A-B): To date, 15 genes have been identified in the human genome that encode at least 26 different alternative transcripts and splice forms representing the B subunits of the PP2A holoenzyme.

As metioned before, these B subunits can be expressed in a tissue specific manner and are proposed to mediate substrate specificity of the PP2A holoenzyme complex.[109] [110]

While the PPP2R1A scaffold can interact with all regulatory B subunits, the PPP2R1B scaffold is unable to interact with the B/PR55 family of B subunits and shows a preference for binding to the B".[108] Assuming that there are no significant differences between PPP2CA and PPP2CB, around 30 PP2A holoenzyme combinations are possible, taking into account the inability of PPP2R1B to interact with the B/PR55 family of B subunits[108] and not including predicted splice variants. Functionally, the number of holoenzymes might even be lower since different subunits within a family might be functionally redundant or expressed in a tissue specific manner under different promoters. Conversely it could be higher since not all regulatory subunits are necessarily identified.

The B subunits have been subdivided into four distinct families (Figure 8) which share no sequence similarity, apart from a few conserved amino acids that allow the interaction with the N-terminal HEAT domains of the PP2A-A scaffold subunit.[111]

According to the subunits we focused in our research work, we are going to explain two out of the four PP2A-B families:

PPP2R2A or PR55α: It belows to the B/PR55 family which consists of at least six members, transcribed from four different genes with more variants likely to exist.[101] PR55 family members exhibit both temporal and spatial expression patterns[112] with both PPP2R2A and PPP2R2C being expressed almost ubiquitously while PPP2R2B and PPP2R2C being highly enriched in the brain. The different PR55 family member proteins also show distinct spatial distribution in the cell.[113] However, whether this variable localization is a regulatory function of these subunits or it simply represents the location of their binding partners, is unclear.

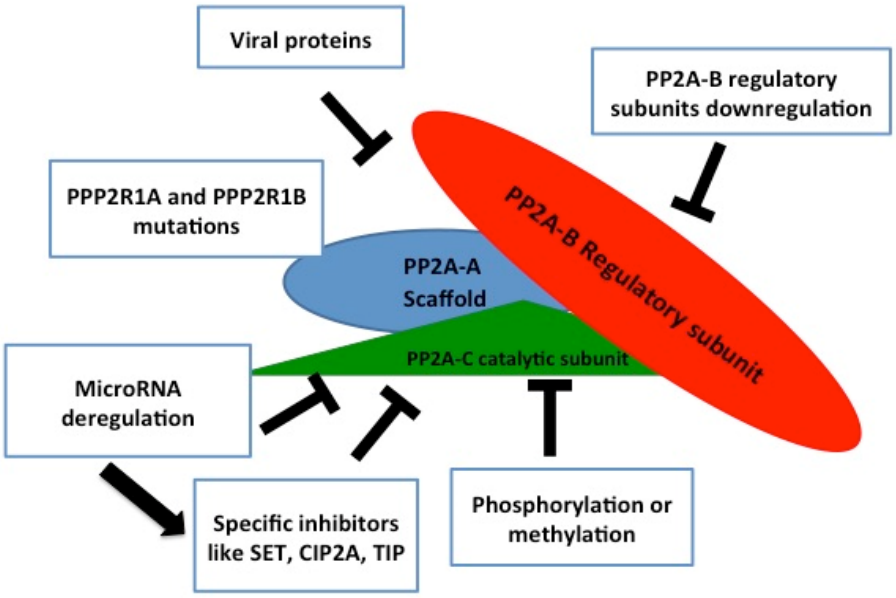
Substrate regulation by B/PR55 family members appears to be dependent on a number of factors.[101] First, substrate binding by the PR55 family members appears to be dependent on a stretch of five degenerate WD40 repeats wich are conserved 40 amino acid sequences that end with a characteristic tryptophan-aspartate that appears to directly mediate protein–protein interactions. Secondly, the PP2Ac subunit of the core enzyme needs to be methylated on Leu309 and dephosphorylated at Thr304 for its interaction with the B/PR55 regulatory B subunits. However, these PP2Ac modifications are more likely to be a consequence of the spatial distribution of the core enzyme rather than anything else.[101]

PPP2R5E or PR61ε: It belongs to the B family of B subunits. The PR61 family of regulatory subunits contains eight members represented by five different genes[114] showing diverse tissue distribution.[115] The PR61 family members show distinct spatial distribution inside the cell with PPP2R5A, PPP2R5B, and PPP2R5E being expressed in the cytoplasm, while PPP2R5C subunits are expressed in the nucleus.

PP2A regulation

Given the complexity of PP2A, the assembly of each specific PP2A complex is tightly and precisely regulated, in part through specific post-translational modifications.[103] Moreover and as mentioned briefly before, the structure of PP2A can be modulated by miR deregulation, mutations affecting the structural subunit, methylation, phosphorylation of PP2A-C at Tyr307 and by alterations affecting the expression levels of PP2A-B subunits. The phosphorylation events result in inactivation of PP2A.[116] In addition, several factors bind to PP2A complexes and affect its phosphatase activity.[101] Factors such as the viral proteins directly inhibit PP2A through displacement of regulatory subunits and similarly, PP2A endogenous inhibitors are able to suppress PP2A activity.[117] [118] Currently, some specific endogenous inhibitors have been reported in mammalians such as SET, CIP2A and TIP (type 2A- interacting protein) (Figure 10).[119] [120]

Figure 10. PP2A regulation



PP2A physiological functions

While most signaling pathways require a cascade of events that are usually carried out by activation of specific kinases, such signals are counteracted by a limited number of phosphatases.[99] PP2A is a phosphatase that functions to reverse the action of kinases in most major signaling cascades.

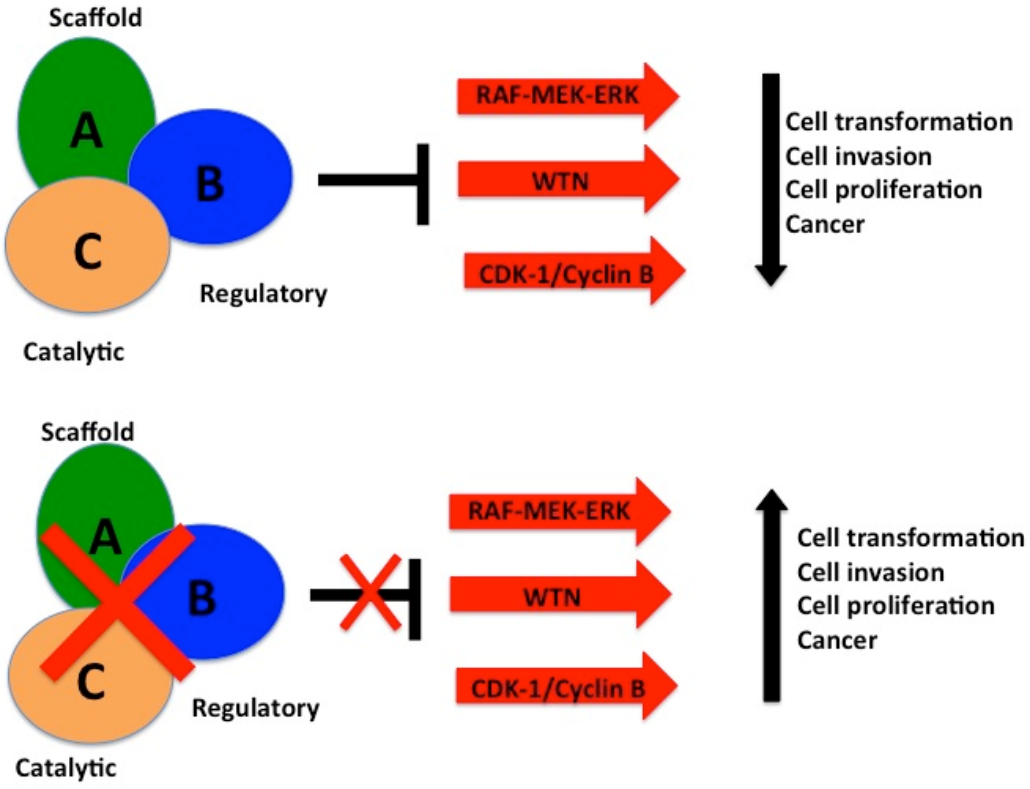
PP2A has a major role in the maintenance of normal cell division.[121] Indeed, PP2A controls the G1/S transition. PP2A also positively regulates mitotic exit through inactivation of Cdk1 and it is also involved in the negative regulation of cell survival and proliferation.[99] Moreover, specific PP2A complexes inhibit anti-apoptotic and mitogenic signals by dephosphorylating and inactivating MEK1 and ERK-family kinases, decreasing the stability and function of transcription factor such as c-MYC and STAT5.[122]

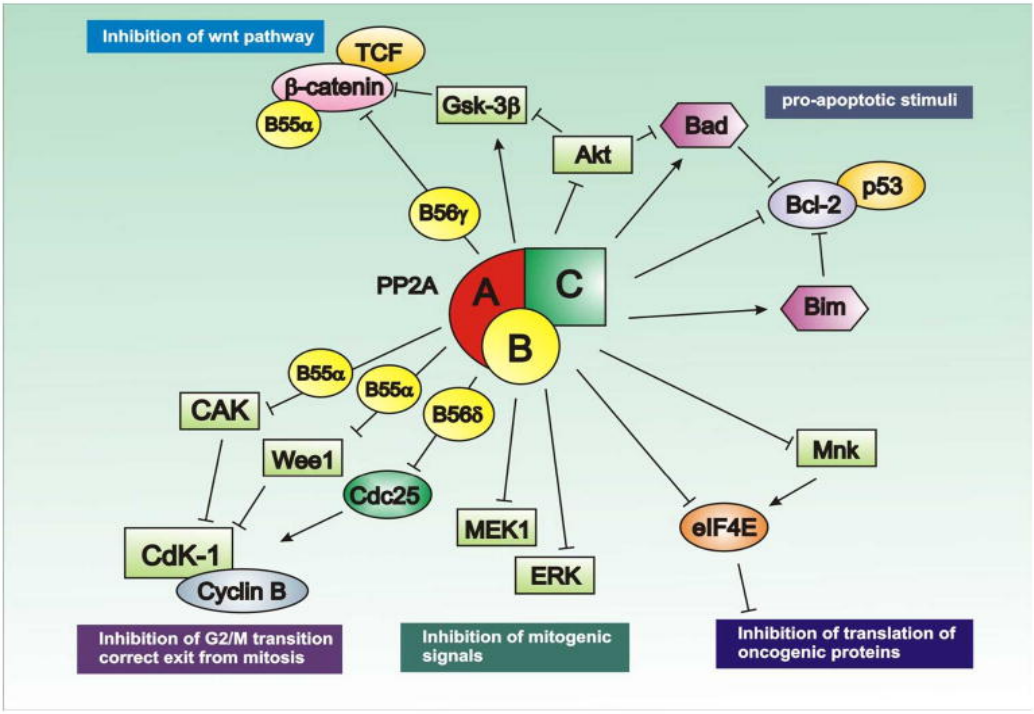
In the same way, PP2A inhibit cap-dependent translation of oncogenes such as Mcl-1 and c-MYC through indirect and direct dephosphorylation of eIF4E (eukaryotic initiation factor 4E).[123]

According to its role as a tumor suppressor, PP2A has pro-apoptotic activity and the ability to negatively regulate the PI3K/Akt pathway by direct Akt dephosphorylation, inactivation of the antiapoptotic Bcl-2, and activation of the proapoptotic factors BAD and Bim.[99]

Finally, PP2A also has an important role in the regulation of the Wnt pathway and, thus, to cell growth and stem cell survival/self-renewal (Figure 11).[101]

Figure 11. Physiological functions of PP2A





(Adapted from Perroti D and Neviani P, Lancet Oncol 2013;14(6):229–38)

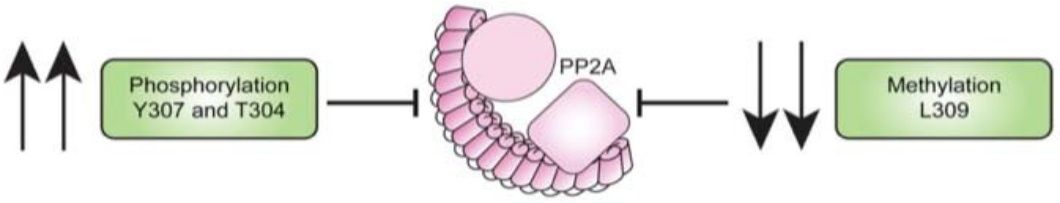
PP2A in cancer

The potential role of PP2A in cancer was reported two decades ago when it was published that okadaic acid, a PP2A selective inhibitor had important tumor-promoting activity.[101] Similarly, it was found that several tumor-promoting viruses are capable of displacing the B regulatory subunits from the core enzyme.[124] The alteration of PP2A by viral proteins leads to the deregulation of similar pathways found disturbed by okadaic acid.

Acummulating evidence established the important role of PP2A as a tumor suppressor gene. Based on the complexity of PP2A function, structure and regulation, it is clear that the role of PP2A as tumor suppressor may be largely cell context and subunit dependent. Thus, the loss of a specific PP2A activity by many different mechanisms, represent a major event contributing to cancer development and progression.[125]

Between the mechanisms that cancer cells use to inhibit PP2A activity are the downregulation of several PP2A subunits, the presence of mutations or genetic alterations affecting some subunits, the deregulation of some miRs, the phosphorylation or methylation of the catalytic subunit (Figure 10 and Figure 12) and the activation of several PP2A endogenous inhibitors.[99]

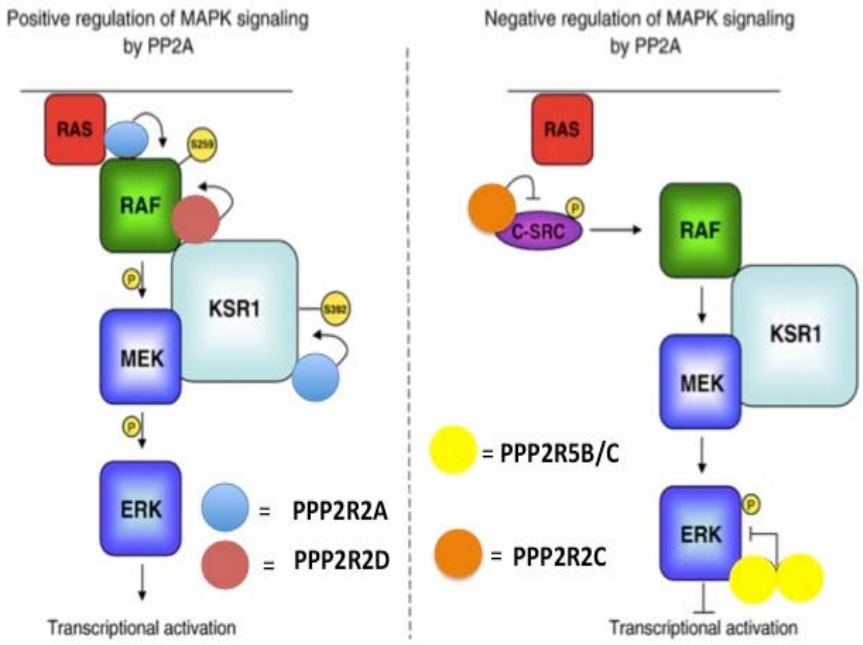
Figure 12. Post-traslational modification of PP2A



(Adapted from Sangodkar J et al, FEBS J 2016;283(6):1004–24)

Moreover, PP2A has been reported as an important regulator of several signaling pathways related to cancer such as MAPK (Figure 13).[101]

Figure 13. PP2A and MAPK pathway



(Adapted from Eichhorn et al, Biochim Biophys Acta 2009;1795(1):1–15)

PP2A and MAPK signaling pathway: Upon stimulation RAS promotes binding of PPP2R2A to KSR1 and RAF leading to the dephosphorylation of Ser392 and Ser295, respectively. This results in the loss of 14-3-3 inhibition and the subsequent recruitment of RAF to the plasma membrane facilitating the RAS/RAF interaction. Similarly, PPP2R2A can dephosphorylate key inhibitory 14-3-3 sites on KSR1 allowing KSR1 to act as a scaffold protein permitting the activation the signaling cascade. RAF can also be dephosphorylated at Ser295 by PPP2R2D leading to downstream ERK activation.[101] In contrast, PP2A can negatively regulate MAPK signaling by inhibiting the kinase activity of c-SRC.[104] c-SRC activates RAF independently of RAS leading to ERK activation. Furthermore, ERK can be directly dephosphorylated by PPP2R2B and PPP2R2D resulting in decreased ERK activation (Figure 13).[126]

Furthermore, loss of PP2A activity has been described in many types of cancer. For example, expression of one of the PP2A regulatory subunit was reduced in melanomas.[127] Similarly, PPP2R2A protein expression was inhibited in a large cohort of AML patient and this correlated with a loss of complete hematologic remission.[128] Interestingly PPP2R2A was found deleted in a subset of luminal B breast cancers.[129] The scaffold PPP2R1A has also been found altered in numerous malignancies. Mutations of the PPP2R1B isoform have been detected in 15% of primary lung tumors, 6% of lung tumor-derived cell lines, 15% of primary colorectal carcinomas, and 13% of breast cancers.[101] Loss of heterozygosity at the PPP2R1B locus was detected in breast, ovary, cervix, stomach, bladder carcinomas and melanoma and aberrant transcripts were found in 29% of HCC[130] and furthermore, PPP2R1B alternative splicing or gene deletion were associated with reduced PP2A activity in aggressive B-CLL.[131] Similarly, PPP2R1A alterations were detected in 9.1% of type I ovarian tumors; 6.7% of type I uterine carcinomas; 19.2% of type II uterine (serous) carcinomas; and, 43% of primary human gliomas.[132]

Phosphorylation of PP2A-C (catalytic subunit) at Y307 has been described as a molecular PP2A-inactivating mechanism with relevance in chronic and acute leukemias.[99 133]

Like those examples, other cancers including both hematological malignancies and solid tumors have been correlated with the loss of PP2A activity (Table 6).[126]

Table 6. PP2A subunit alterations and malignancies

Subunit	Gene	Disease
A	*PPP2R1A*	Breast, lung, melanoma, ovarian, endometrial, uterine, colon
		Breast
		Glioma
A	*PPP2R1B*	Breast, colon, lung
		Breast
		Breast, lung, ovarian, cervical, melanoma, NHL, CLL
		AML
		HCC, B-CLL
B	*PPP2R2A*	Breast, prostate, mieloma
		AML
B	*PPP2R2B*	Breast.
B	*PPP2R2C*	Breast, prostate
B’	*PPP2R5A*	Melanoma
B’	*PPP2R5C*	Melanoma
		Lung
B’	*PPP2R5E*	Soft tissue sarcoma
C	*PPP2CA*	AML, prostate

However, before the research works concerning the present doctorate thesis, little had been explored about the relevance of the loss of PP2A activity in CRC. In this regard, before year 2012, only a few investigations were done looking for PP2A role in CRC. The little evidence about the role of PP2A in CRC was for the article published by Yuan Q et al[134] which suggest that decreased expression of PP2A in CRC cell lines may play a role in CRC pathogenesis. Also, Junttila et all in 2009[119] reported that the loss of activity of PP2A via CIP2A was related to human malignancies including CRC and showed biochemical evidence that PP2A is an important regulator of Wnt/beta-catenin pathway activity in these CRC cells.[135]

Oncoprotein SET: an endogenous PP2A inhibitor

SET structure and regulation

SET oncoprotein also named I2PP2A, inhibitor 2 of PP2A, is transcribed from the SET nuclear proto-oncogene (TAF-1β/I2PP2A). It is a 39-kDa phosphoprotein widely expressed in human tissues and found predominantly in the cell nuclei, although it has been also found associated to the endoplasmic reticulum.[136]

SET is a binding partner of PP2A with inhibitory function (Figure 10). It was identified as an oncogene found fused to CAN in t (6; 9) acute undifferentiated non-lymphocytic leukemia and also fused with the nucleoporin Nup-214a in adult and pediatric T-ALL patients carrying the cryptic and recurrent deletion del(9)(q34.11q34.13).[137 138]

SET can be modulated for transcription factors and miRs. For instance, the transcription factor EVI-1 and the miR-199b-5p have been described to regulate SET expression in AML and choriocarcinoma.[125 139]

SET physiological functions

SET protein has multiple cellular functions including control of cell cycle,[136 140] gene transcription,[141] apoptosis, cell migration[142] and epigenetic regulation such as chromatin remodelation.[143]

As mentioned before, SET oncoprotein, is a potent physiologic inhibitor of PP2A.[101] Physiologically, SET protein directly binds with PP2A-C and inhibits PP2A phosphatase activity.[143 144]

SET is also able to suppress the DNase activity of NM23-H1 tumor suppressor, deregulates AKT signaling, negatively regulates p53 acetylation repressing its activity, increase AP-1 activity, activate MAPK signaling, and regulate granzyme B and interferon-γ production in human natural killer cells.[145-146] Importantly, SET is also able to control c-MYC.[147]

SET in cancer

SET plays an oncogenic role modulating signaling pathways with high relevance in human cancer. Furthermore, SET expression is high in rapidly dividing cells, but low in quiescent or contact-inhibited cells, indicating a potential selectivity for SET expression levels and cell growth potential.[148] SET also forms an inhibitory complex with NM23-H1,[149] whose expression level is inversely related to the metastatic potential of a cancer cell. Finally, SET overexpression is able to induce c-Jun and AP-1 activities thereby contributing to the malignant phenotype.[150]

This protein has been correlated with the pathogenesis of several cancers: SET is highly expressed in Wilms' tumors[148] and can contribute to liver carcinogenesis.[151] Moreover, several studies showed that SET was up-regulated in ALM,[125] head and neck squamous cell carcinoma,[152] and breast cancer.[153-154] SET was also overexpressed in BCR-ABL1^{+} leukemias and interestingly overexpression of SET is a poor prognostic factor in AML patients, in primary B-CLL cells, and B-cell non-Hodgkin lymphoma cell lines.[117]

It has been reported that the antitumor effects showed by the PP2A activator FTY720 are mediated by SET in lung cancer.[155] Indeed, FTY720 (Fingolimod/Gilenya®), originally approved for its use in multiple sclerosis by Novartis, activates PP2A via inhibition of SET. FTY720 was derived from a fungal metabolite and acts as an immunosuppressant by modulating the

sphingosine-1- phosphate receptor. FTY720 exerts antitumor activity in breast, HCC, glioma, and multiple myeloma models. Specifically in chronic myeloid leukemia activation of PP2A by FTY720 induces apoptosis through the inactivation of BCR-ABL1 and negative regulation of several survival factors including ERK.[126]

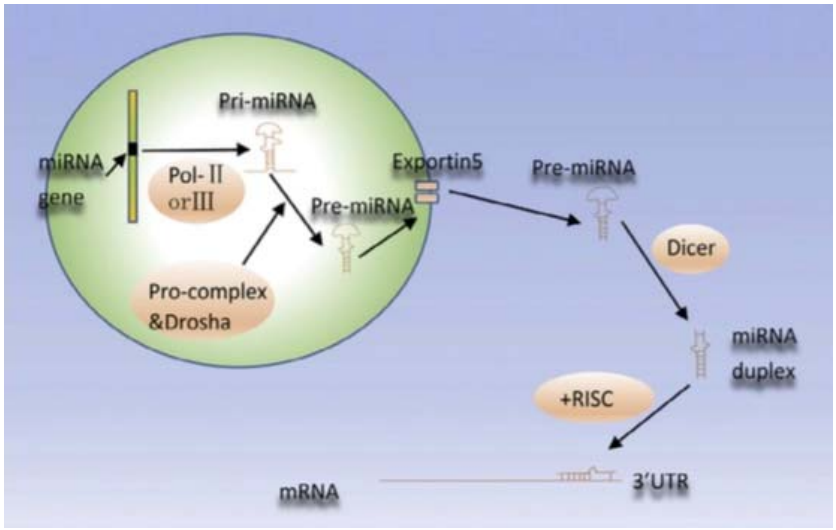
Before our research work, little was known about the role of SET in CRC, the clinical potential as a biomarker and the biological effect of its overexpression.

MicroRNAs: What are they and why are they important?

Since the identification of miRs in 1993, and the subsequent discovery of their highly conserved nature in 2000, the amount of research into their function—particularly how they contribute to malignancy—has greatly increased (Figure 16). MiRs are 19-24 sequences of nucleotides (nt) long and serve as major regulators of gene expression through their ability to bind and post-transcriptionally mediate the expression of targeted messenger RNAs.[156]

MiRs are translated by polymerase II (or less frequently polymerase III) to form pri-miRNA, composed of hundreds of nt, including a 33 bp stem and a terminal loop structure with flanking segments. Next, pri-miRNA is transformed into pre-miRNA by a protein complex including Drosha to generate a 60–70 nt long hairpin RNA with 2 nt overhangs at its 3'-end. Pre-miRNA is then transported by exportin-5 from the nucleus to the cytoplasm, and combines with Dicer. The miRNA duplex now contains the mature miR guide and its complementary passenger strand (miR*). Per the widely accepted model, miR* is degraded, although evidence suggests miR* may also be functional as well. The single remaining strand integrates into the RNA- induced silencing complex (RISC), a ribonucleoprotein effector containing a catalytic endonuclease core, Ago2, and binds the site of 3'-untranslated regions (3'-UTR) in target mRNA. In turn, the "seed region" (the 5'-end region of mature miR, which includes 2–8 nt) binds the 3'-UTR of target mRNA (Figure 14).[157]

Figure 14. MiR processing pathway



(Y Xuan et al Cancer Letter 2015;360(2):89–105)

According to the match level, the target mRNA will be degraded or blocked but not decreasing its expression level. Most animal miRs are imperfectly complementary, resulting in translation inhibition of target mRNA rather than full blockage.[157] Thus, it is important to

realize that the miRs dependent regulation of gene function may vary from an “on/off switch” to much milder changes in expression levels.

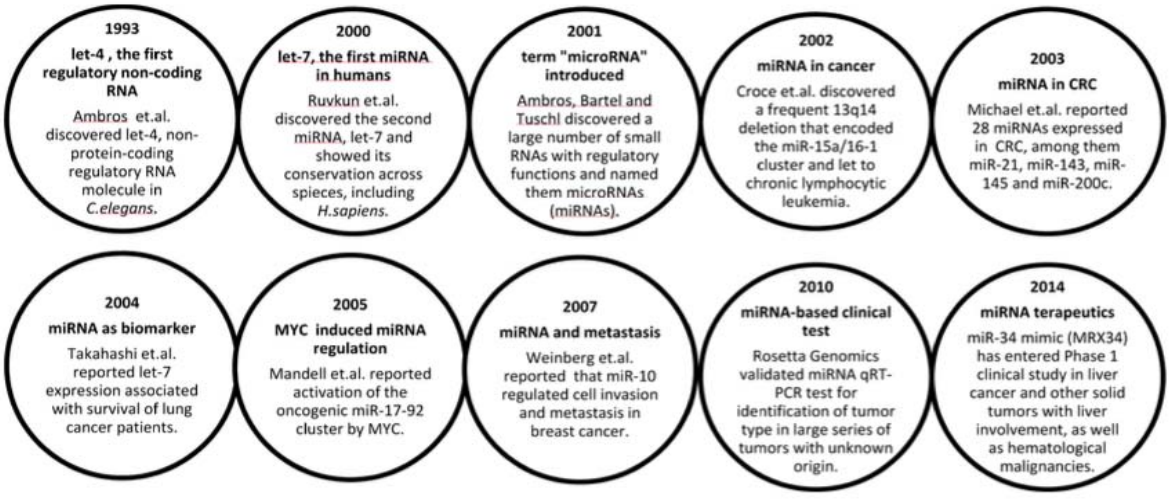
To date, thousands of miR precursors (pre-miRs) and mature miRs have been identified.[158] This issue is based on the fact that each miR can regulate dozens or even hundreds of genes, affecting the activity of entire pathways and networks. On the other hand, one gene is often regulated by several different miRs and can have up to 50 miR binding sites.[31] Indeed, miRs are predicted to regulate approximately 60% of the human genes.[158]

MicroRNAs and cancer

According with their function, miRs are particularly influential in pathologic processes like proliferation, differentiation, apoptosis, invasion, angiogenesis and metastasis via gene expression manipulation.[156]

In 2002, the first report about the role of miRs in cancer established that the gene cluster containing the miRs miR-15 and miR-16 is deleted in most people with CLL.[159] Further studies have shown that miR-15 and miR-16 act as tumour suppressors by targeting the oncogene BCL2, which encodes a protein involved in cell survival.[160] MiRs relevance in tumoregenis and their potential clinical impact have been progressively growing. In fact, in 2014, started the first phase I clinical trial that use miRs as a tool for cancer therapeutics (Figure 15).[161]

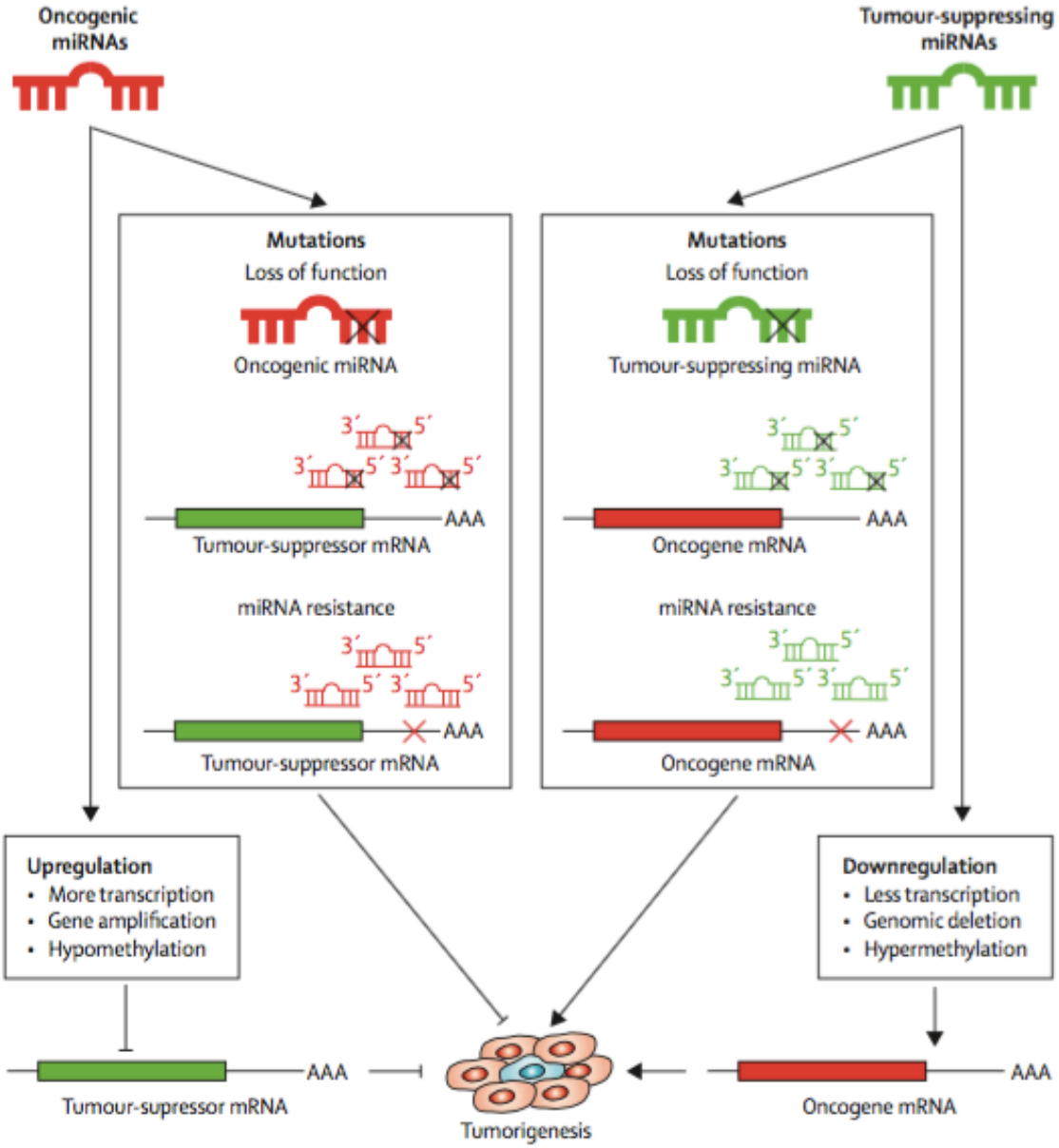
Figure 15. Milestones in miRs discovery realted to cancer



(Cekaite et al, Oncotarget 2016;7(6):6476–505)

They can act as tumor suppressor or oncogenes depending on their target mRNA.[156] Thus, upregulation of oncogenic miRs reduces expression of tumor-suppressor protein, but downregulation of tumor suppressor miRs results in an increased production of oncogenic proteins. Loss-of-function mutations in oncogenic miRs and mutations in tumor suppressor mRNA would increase expression of tumor-suppressor proteins and hence reduce tumorigenesis (Figure 16).

Figure 16. Regulation of tumorigenesis by miRNAs



(Kong et al, Lancet Oncol 2012;13(6):249-58)

Modulation of miR expression is thought to be an important mechanism by which tumor suppressor proteins and oncoproteins exert some of their effects. For instance, the proto-oncogene c-MYC transcriptionally activates the miR-17-92 cluster.[162] c-MYC also represses transcription of many tumour-suppressor miRNAs, including the let-7 family.[163] In the same way, reduced expression of let-7 miRs is recorded in many cancers,[161] and correlates with poor survival. The let-7 family targets the enzyme RAS, which is involved in cell growth, differentiation, and survival.[164] Moreover, the tumour suppressor P53 transcriptionally induces the miR-34 family in response to DNA damage and loss of miR-34a expression is associated with metastasis and recurrence of prostate cancer[165] and expression of miR-34b and miR-34c is lost through deletion or hypermethylation, or is downregulated, in 90% of CRCs.[166]

However, gene regulation is not the only way in which miRs are implicated in malignancies. Mutations of miRs binding sites in the 3'untranslated region (UTR) of oncogenes are correlated

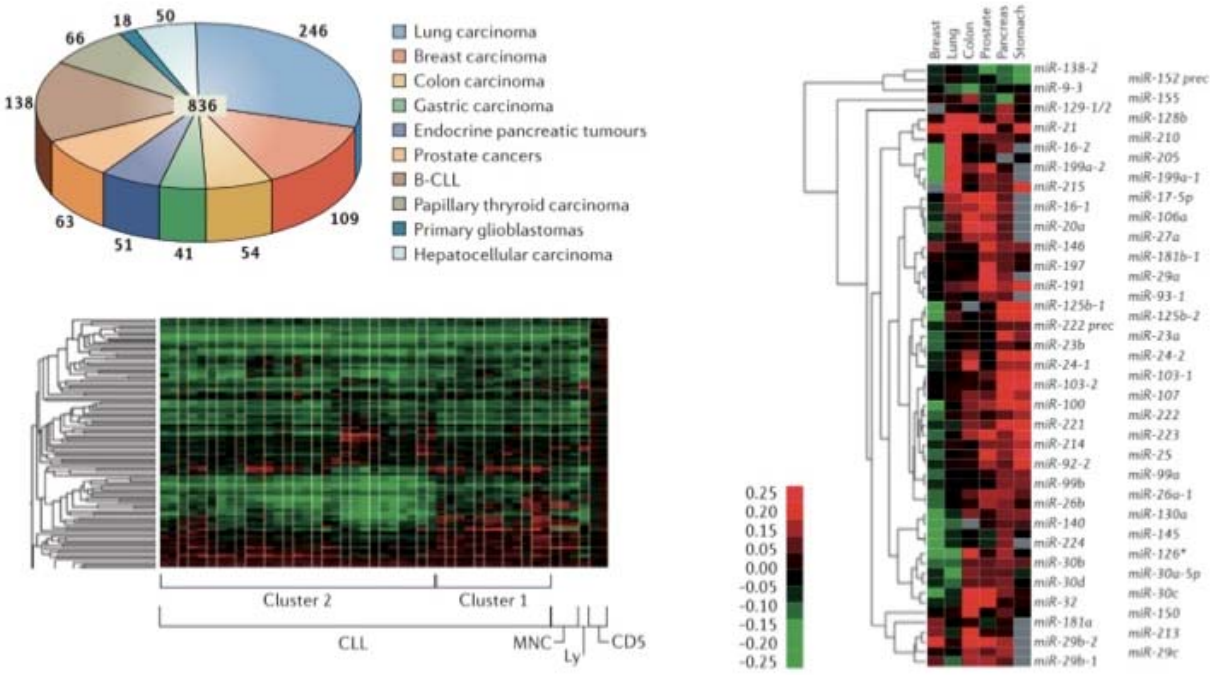
with an increased risk of cancer. For example, a single nucleotide poly-morphism in the 3'UTR of the KRAS oncogene significantly increases risk of non-small-cell lung cancer.[167]

MiRs can also control the activity of cancer stem cells. Some miRs involved in stem-cell regulation are miR-296, miR-134, miR-470, and the miR-34 family, which targets genes essential for pluripotency and stem-cell function as, Oct4, NANOG, SOX2, NOTCH, and BCL2.[168]

Moreover, miRs are important controling angiogenesis. Promotion of angiogenesis by tumours necessitates activation of proliferation and migration pathways in vascular smooth muscle cells. For instance, in nasopharyngeal carcinoma cells, the miR-15a/16-1 cluster regulates angiogenesis by targeting the angiogenic factors VEGFA and MET.[169]

Apart from being essential for all the tumor related process above mentioned, miRs expression profiles are able to classify human cancers. Thus, miRs profiling has shown significantly different miR profiles compared with normal cells from the same tissue (Figure 17).

Figure 17. MiRs profile in human cancers



(Adapted from Calin GA et al, Nat Rev Cancer 2006;6(11):857-66)

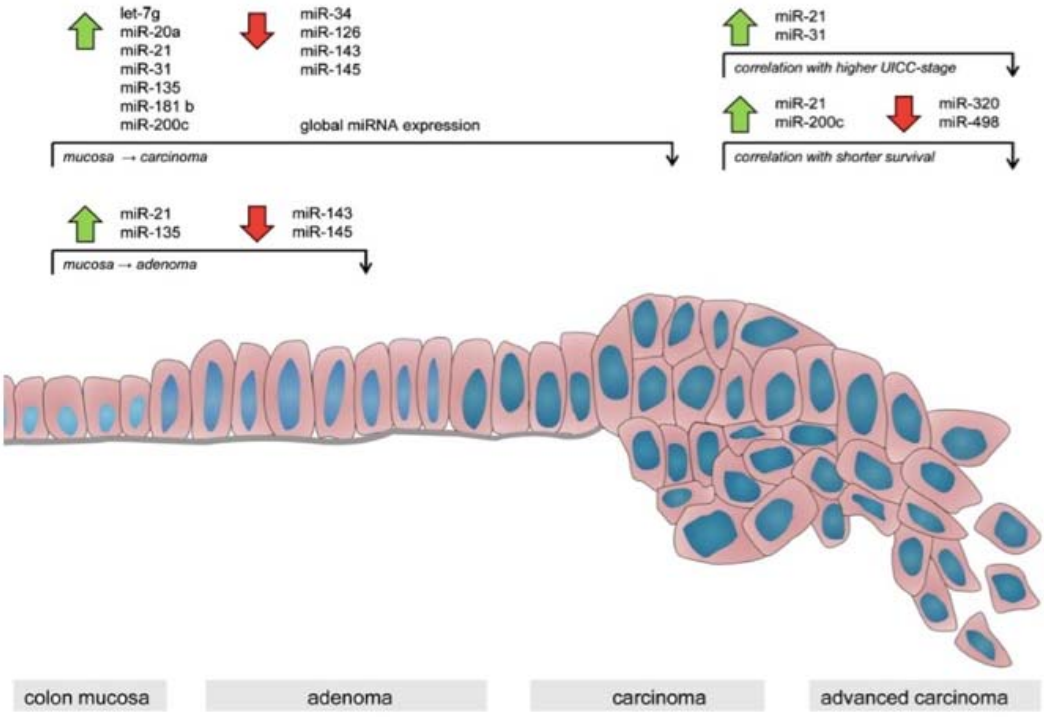
MicroRNAs and CRC

As metioned before, CCR development, progression and therapy response are controlled by genetic and epigenetic events. Epigenetics refers to the study of mechanisms that alter gene expression without permanently altering the DNA sequence. Those epigenetic changes involve histone modification, DNA methylation and non-coding RNA-mediated gene silencing including miR alterations.[31]

Recent advances in the study of miRs have not only offered a deeper understanding of the underlying mechanisms of CRC carcinogenesis, but also allowed identification of clinically relevant putative biomarkers for disease monitoring.[156 170]

Several miRs have been reported to participate during each step of the normal-adenomatous polyp-cancer of CRC development (Figure 18).

Figure 18. MiRs and CRC development



(Faber C et al, Virchows Arch 2009; 454(4):59-367)

For example, the miR-17-92a cluster, miR- 143, miR-135b, and miR-145 regulate WNT/β-Catenin signaling pathway, which is involved in CRC initiation.[171-172] Moreover, genes associated with the PI3K/AKT and RAS-MAPK cascades, that drive the transition from early to advanced adenoma, are controlled by specific miRs (for instance, RAS-MAPK by miR-143, let-7, miR-21, and miR-31; and PI3K/AKT by miR-1, miR-21 and miR- 143).[37 173-174] In the same way, p53, frequently inactivated during the evolution from advanced adenoma to adenocarcinoma, is controlled by miR-34a/b/c, miR-133a, miR-143, and miR-145.[175] In addition, miR-21, miR-155 and miR-200 family members regulate the TGF-ß pathway.[156 176 177]

The available data about miRs and CRC is growing quickly. In fact, approximately 450 miRs have been associated with CRC, of which twenty account for one third of all miRNA quotations in pubmed. Of those, miRNAs miR-21, miR-143 and miR-145 were the most frequently reported, followed by miR-31, miR-34a, miR-200c, miR-20a and miR-92a.[161]

MiRs as Diagnostic Biomarkers in CRC

The first systematic and comprehensive miR expression profiling study was conducted by Ng and colleagues,[178] who evaluated miR expression alterations in tissue and plasma samples from CRC patients and healthy subjects. This study revealed that high expression of two miRs,

miR-92a and miR-17-3p, could discriminate patients with CRC from healthy subjects. Moreover, this landmark study further reported that the plasma levels of both miRs decreased significantly following surgical resection of the primary tumors. Later, their findings have been replicated in other studies including CRC patients.[31 179]

Furthermore, miR-21 is considered one of the promising biomarkers for the early detection of CRC owing to the following attributes: dysregulation of miR-21 occurs frequently in early stages of the adenoma-carcinoma sequence;[180] miR-21 is one of the most highly expressed miRNAs in CRC;[181] and miR-21 is highly secreted by cancer cells and can be measured in exosomes or as free miRNAs in plasma or serum.[182 183]

Although increasing number of miRNAs have been identified as potential biomarkers for early diagnosis of CRC,[179 184 185] it seems unrealistic that a single miR will adequately capture the underlying molecular heterogeneity in colorectal polyps and cancers. Accordingly, several studies have proposed combining miRs into a biomarker panel to improve the detection accuracy for colorectal neoplasms.[186 187]

MiRs as Prognostic Biomarkers in CRC

In 2008, a study that used a microarray-based approach to evaluate the expression levels of 389 miRNAs in 84 CRC and matched normal colonic tissues was performed.[180] This study identified and validated 37 differentially expressed miRNAs, including miR-20a, miR-21, miR-106a, miR-181b and miR-203. A finding of this study was that high expression of miR-21 in CRC patients is associated with poor survival, which has since been independently confirmed in several other reports.[182 188 189] Although several other overexpressed (miR-10b, miR-17-92a cluster, miR- 29a, miR-31, and miR-182)[190 191] and underexpressed miRNAs (miR-143 and miR-124)[31] have been proposed to be prognostic biomarkers, currently miR-21 has the best potential to be a clinically useful miRNA-based prognostic biomarker in CRC.

Moreover, Schepeler and colleagues[192] found that miR-320 and miR- 498 miR- distinguish high-risk from low-risk stage II patients and correlate with recurrence-free survival. In addition to individual markers, a miR panel has been developed to identify stage II CRC patients with a high risk of recurrence. At this research work, the expression of 1849 miRs in 40 paired stage II colon tumors and adjacent normal mucosa tissues was examined[189] resulting a miR-based classifier comprising of miR-20a-5p, miR-21-5p, miR-103a-3p, miR- 106a-5p, miR-143-5p and miR-215 was developed, which discriminated high risk in stage II CRC patients.

MiRs as predictive Biomarkers for Response to Treatment in CRC

Currently, the majority of the results related to miRs role in drug resistance are based on in vitro studies and remain to be assessed in clinical sample sets. MiRs shown to mediate 5-fluorouracil resistance include miR-10b, miR-19b, miR-20a, miR-21, miR-23a, miR-31, miR-34, miR-129, miR-140, miR-145 miR-192/-215, miR-200 family, and miR- 497.[31] MiRs have also been identified that mediate irinotecan resistance (miR-21 and miR-451)[31] and oxaliplatin resistance (miR-20a, miR-21, miR-133a, miR-143, miR-153, miR-203, and miR-1915).[31] As mentioned before, clinical data supporting these miRNA biomarkers as drug resistance markers are limited or nonexistent. However, a recent study of tumor tissues collected from KRAS wild-type mCRC patients treated with anti-EGFR therapy identified miR-31-3p as a negative predictor of PFS.[193]

MicroRNA-199b-5p

Human miR-199b-5p is located on chromosome 9 and is an intragenic miR encoded in the dynamin 1 (DNM1) gene, from the opposite strand in a 2.2 kb intronic region between exons 14 and 15. Mature 5p arm, encoded by hsa-miR-199b, is evolutionarily conserved among

several species. Transcription of miR-199b-5p is predicted to be independent of its host gene, DNM1.[194]

It has been reported that downregulation of miR-199b-5p plays a central role in cancer progression, development of metastasis and poor prognosis in several cancers, including head and neck cancer,[195] breast cancer,[196] prostate cancer,[197] osteosarcoma,[198] AML,[194] and medulloblastoma.[199] Thus, miR-199b-5p is considered a tumor suppressor miR.

It has been proposed as a SET inhibitor in choriocarcinoma[139] as well as involved in acquired chemoresistance in CML or ovarian cancer.[200] [201] In addition, miR-199b-5p also functions as a tumor suppressor in medulloblastoma through negative HES1 regulation,[202] hepatocellular carcinoma and prostate cancer by targeting HIF1α[203] [203] and breast cancers by affecting HER2.[204]

In spite of the accumulating evidence highlighting the importance of miR-199b-5p in human cancer, before our study, no study had investigated the role of miR-199b-5p in CRC.

MicroRNA-31

The gene encoding miR-31 is located on chromosome 9p21.[205] MiR-31 is among the most frequently altered microRNAs in human cancers and altered expression of miR-31 has been detected in a large variety of tumor types both as a tumor suppressor miR and as a oncogenic miR, typically depending on the cancer type.[206] That is one issue that makes miR-31 especially interesting among the miRNAs deregulated in cancer. Increased expression of miR-31 has been detected in colorectal, lung and pancreatic cancer, head and neck squamous cell carcinoma and osteosarcoma.[207]

In CRC, miR-31 overexpression has been demonstrated in primary tumors across all clinical stages as well as in colon cancer cell lines.[190] [208] [209] MiR-31 plays an important role in regulation of cancer cell growth, mobility, and other cancer associated functions. As mentioned before, it role as diagnostic, prognostic and predictive biomarker in CRC is increasing and perhaps the most interesting of these is the link to therapy resistance because in colon cancer, inhibition of high endogenous miR-31 expression sensitized cancer cells to 5-FU[210] and miR-31 overexpression seems to predict lack of response to EGFR inhibitors like cetuximab.[211] However, before starting the present research work, the role of miR-31 determining resistance to 5-FU based chemoradiotherapy in LARC had not been explored.

MiR-31 has been proven to regulate the expression of several genes such as ATN1 (a negative regulator of transcription) and RASA1 (a GTPase activator) in CRC.[206] Furthermore, one of the groups of validated miR-31 target genes are those involved in the regulation of cell proliferation and apoptosis. Thus, miR-31 was demonstrated to act in lung cancer as an oncogenic miR through targeting PPP2R2A.[212]

MicroRNA-21

Human miR-21 is highly conserved among species including human, rat, mouse and frog. It is located on chromosome 17q23 overlapping the TMEM49 gene. MiR-21 encodes a single hairpin and is regulated by its own promoter containing binding sites for AP-1 and PU.1 transcription factors.[213] Experimental data has shown that miR-21 functions in many cell types as an anti-apoptotic and pro-survival factor and plays a significant role in many cancer biology and prognosis.[214]

As mentioned previously, miR-21 is a well studied oncogenic miR with an important role in CRC pathogenesis.[31] [182] [215] A number of miR-21 target genes have been previously identified in CRC including PDCD4, RhoB protein and PTEN.[216] Also, miR-21 expression is regulated by the

STAT3 and NFkB transcription factors, which are both constitutively activated in a variety of cancers and play critical roles on tumorigenesis.[217]

Moreover and as described previously, miR-21 importance as a non-invasive biomarker in CRC is increasing. Indeed, miR-21 is probably the most relevant biomarker for diagnosis and prognosis reported in CRC. One of the first studies using miRNA expression profiling of primary CRC tissues and the adjacent normal mucosa identified miR-21 as differentially expressed in CRC.[31] Later, validation studies in an independent set of plasma samples demonstrated that plasma miR-21 could be used to discriminate patients with CRC from normal control patients with high sensitivity (90%) and specificity (90%). Furthermore, the diagnostic and prognostic potential of serum miR-21 in CRC patients was addressed in another study, which revealed that tumor-derived circulating miR-21 expression not only accurately discriminated patients with CRC from healthy subjects, but allowed identification of patients with advanced adenomas, which are target lesions for an ideal CRC screening test.[182] This study also confirmed the significant association between lower miR-21 expression in serum and CRC tissues following curative resection of the primary tumor. Consequently, several studies confirmed the potential of miR-21 for use as a single miR biomarker for the early detection of CRC.[218]

However, before the present research, the role of miR-21 determining the efficacy of 5-FU based CRT in LARC was not known.

RESEARCH HYPOTHESIS AND OBJECTIVES

Research hypothesis

CRC is a high prevalent neoplasia characterized by a progressive accumulation of genetic and epigenetic abnormalities that led to cancer progression. Despite recent advances in the molecular CRC landscape with relevance on currently CRC clinical management and with a significant impact improving OS, prognosis of stage III and IV CRC is still poor. Furthermore, the newest line of molecularly targeted therapeutic agents, appear to only have activity in mCRC and do not cure the patient, but they have exponentially increased treatment costs and the economic burden of CRC care. Thus, a better understanding of CRC molecular alterations that allow the finding of new robust biomarkers to guide CRC management and to guide the election of new molecular target therapies are clearly and urgently needed to improve OS, management and quality of life of patients with metastatic or locally advanced CRC.

PP2A have been progressively considered a key tumor suppressor gene and its inactivation has been described as a common event in the cancerous cells through several molecular strategies such as alterations of its endogenous inhibitors, phosphorylation of the PP2A-C, alterations affecting any subunit and/or miRs deregulation. However, PP2A potential clinical and therapeutic relevance in CRC remains poorly studied.

Furthermore, the standard of care for LARC involves a multidisciplinary approach consisting of neoadjuvant chemoradiotherapy based on 5-FU regimens followed by total mesorectal excision surgery. However, not all the patients have a good response leading to unnecessary toxicities and surgery delays. MiR-21 and miR-31 are two of the most significantly deregulated miRNAs in CRC. Overexpression of both of them has been associated with and oncogenic role and resistance to 5-FU in the CRC. Moreover, there is some evidence that miR-21 and miR-31 could be modulating PP2A activity. However, miR-21 and miR-31 ability to predict neoadjuvant CRT based of 5-FU pathological response and outcome in LARC was a question that remained to be explored.

Therefore, and based on the above mentioned we hypothesized that PP2A deregulation would be an important event in CRC. Thus, we planned a study to investigate the effects of PP2A inhibition in CRC, the molecular strategies that the cancer cells use to inhibit PP2A in CRC and the potential clinical relevance of PP2A inhibition in CRC. Moreover, we hypothesized that miR-21 and miR-31 could serve to predict response to the currently LARC standar treatment.

Research objectives

1) **To determine the status of PP2A in CRC** analyzing both clinical samples and CRC cell lines.
2) **To assess the biological effects of PP2A inhibition in CRC** performing functional in vitro studies using several CRC cell lines.
3) **To analyze the molecular mechanism of PP2A inactivation in CRC** by analyzing PP2A endogenous inhibitors, some alterations of specific PP2A subunits and miR deregulation. For that aim, we plan to analyze the presence of PP2A inhibitory alterations in clinical samples and perform functional in vitro studies in several CRC cell lines.
4) **To identify novel biomarkers with prognostic and predictive value in CRC based on PP2A inhibition** using four independent CRC patient cohorts.

5) **To explore PP2A reactivation as a novel therapeutic strategy in CRC** using PP2A activators such as forskolin and FTY720.

MATERIAL AND METHODS

With the aim of evaluating our research objectives, we planned several in vitro studies and a clinical validation using 4 independent cohorts that compromised a total 522 CRC patients. All of the manuscripts were elaborated following REMARK guidelines for biomarker research (Table 7).[219]

Table 7. REporting recommendations for tumor MARKer prognostic studies (REMARK)

Introduction
1. State the marker examined, the study objectives, and any prespecified hypotheses.
Materials and Methods
Patients
2. Describe the characteristics (e.g. disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria.
3. Describe treatments received and how chosen (e.g. randomized or rule-based).
Specimen characteristics
4. Describe type of biological material used (including control samples), and methods of preservation and storage.
Assay methods
5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point.
Study design
6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease or age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.
7. Precisely define all clinical end points examined.
8. List all candidate variables initially examined or considered for inclusion in models.
9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.
Statistical analysis methods

10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.
11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.
Results
Data
12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.
13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.
Analysis and presentation
14. Show the relation of the marker to standard prognostic variables.
15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan–Meier plot is recommended.
16. For key multivariable analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.
17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their significance.
18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, internal validation.
Discussion
19. Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study.
20. Discuss implications for future research and clinical value.

The details of all the experiments and clinical cohorts are explained as follow. Moreover, they are also described in the articles published that derive from this doctorate thesis (see Results: scientific publications)

In vitro approach

Cell culture

The human CRC cell lines RKO (ATCC CRL- 2577), SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38), LoVo (ATCC CCL-229), HCT-116 (ATCC CCL-247) and LS513 (ATCC CRL-2134) and SW620 (ATCC CCL-227) were purchased from American Type Culture Collection (ATCC). Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum and were grown at 37ºC in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/mL) and streptomycin (0.1 mg/mL). Cells were, depending of the experiment, treated with one or more of the following reagents: 5-FU (1 µmol/L; Sigma), SN-38 (50 nmol/L; Sigma), Oxaliplatin (1 µmol/L; Sigma), FTY720 (10 µmol/L; Calbiochem), forskolin (40 µmol; Calbiochem) and okadaic acid (2.5 nmol/L; Calbiochem).

Trasfection

For transfection experiments, CRC cells were seeded in 6-well plates and transfected with one or more of the following vectors, depending on the experiment: 10 µL of Lipofectamine 2000 (Life Technologies), 2 µg of plasmidic vectors or 75 nmol/L of SET-specific siRNAs designed and synthesized by Dharmacon RNA Technologies or 20 nM of a miR-199b specific *mir*Vana™ miRNA Mimic and Inhibitor (Ambion). Briefly, the plasmidic DNA and the LipofectAMINE are added to 500 µl RPMI without serum and antibiotics, mixed, incubated at 37 for 20 min and added to the cell culture. Furthermore, the cells at 70% confluence are seeded in 6-well plates with 1.5 ml of antibiotic-free RPMI supplemented with 13% serum. After 4 hours, the medium is replaced with fresh RPMI supplemented with 10% serum and 1% antibiotics.

RNA isolation

Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumor specimens using RecoverAll Total Nucleic Acid Isolation kit (Ambion) before macrodissection of the samples. For that, FFPE samples are incubated in xylene at elevated temperatures to solubilize and remove paraffin from the tissue, then washed in alcohol solutions to remove the xylene. The deparaf-finized samples are next subjected to a protease step to digest protein covalently bound to RNA. Finally, the nucleic acid is purified by capture on a glass-fiber filter, washing and elution. High-ethanol washing steps ensure the recovery of smaller RNA fragments, like miRNA. The steps are explained in detail as follows:

Deparaffinization: we add 1ml of Xylene 100% to all the samples, we mix and we incubate at 60ºC approximately 30 minutes to put out the paraffin. Then, we centrifugate for 2 minutes at maximum speed and discard the xylene, wash the pellet twice with 1 ml 100% ehanol and vacuum or air dry the pellet to remove the residual ethanol.

Protease digestion: we add digestion buffer and protease and incubate at 80ºC for bettwen 4 hours.

Nucleic acid isolation: we prepare the isolation additive/ethanol mixture and add it to the samples and mix. Next, the mixture is passed throught the filter cartridge. After that, we wash with 700µl of wash 1 buffer and later wash with 500µl of wash 2/3 buffer. Finally, a centrifugation is performed to remove the residual fluid.

Nuclease digestion and final purification: We add the RNA mix to each filter cartridge and

incubate for 30 min. Next, we wash with 700µl of wash 1 buffer and twice with wash 2/3 buffer to then, centrigugate to remove the residual fluid. Finally, we elute with 60 µL of Elution Solution or nuclease-free water at room temperature.

Quantification of miR expression levels

Samples were reversed transcribed using the TaqManHMicroRNA Reverse Transcription Kit (Applied Biosystems), and mature miRs were quantified by quantitative real-time RT-PCR using TaqMan MicroRNA Assays (Applied Biosystems) specific for miR- 21, 31 or 199b and U6B as internal control. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta CT}$ method.[220]

Quantification using the TaqMan MicroRNA Assays is done using two-step RT-PCR: In the first step, the reverse transcription step, cDNA is reverse transcribed from total RNA samples using specific miRs primers from the TaqMan miRs assays and reagents from the TaqMan miR Reverse Transcription Kit. In the second one, the PCR step, PCR products are amplified from cDNa samples using the TaqMan miR assay together with the TaqMan Universal PCR master mix.

We need to prepare the total RNA and perform the reverse trasnscription within the thermal cycler. Next, we create and set up a plate document and prepare the PCR reaction plate that is an optical 96-well thermal cycling plate. Later, we run the PCR reaction plate using the applied biosystems 7500 fast Real-Time PCR System. Finally, we performe the analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method.[220]

For the reverse transcription, we synthesize single-stranded cDNA. This process involves preparing the RT master mix, preparing the RT reaction plate and performing the reverse transcription. For the PCR amplification, cDNA are amplified using sequence-specific primers from the TaqMan assay plates. It requires preparing the reaction plate, setting up the plate document and running the plate.

Real-time PCR

cDNA amplication was done in a 7500 Fast Real-Time PCR System (Applied Biosystems) at 40 cycles, with the following temperature conditions: 50ºC 2minutes, 95ºC 10minutes, 40x(95ºC 15seconds – 60ºC 1minute) – hold 16ºC. TaqMan Gene Expression Assays specific for our targets were used. Analysis of relative gene expression data was performed using the $^{2-\Delta\Delta CT}$ method.[220]

Western blot analysis

Protein extracts were isolated using Trizol Reagent (Invitrogen), clarified (12,000 g, 15 minutes, 4ºC), denatured, and subjected to SDS-PAGE and Western blot analysis. The electrophoresis was performed at 80 V for 15 minutes and 120 V for one hour. The transference was performed at 120 V for one hour. Antibodies used were, depending on the work: mouse monoclonal anti-PP2A (clone 1D6, Upstate Inc.), rabbit polyclonal anti-PP2A (FL-309; Santa Cruz Biotechnology), rabbit monoclonal anti-PP2AY307 (Epitomics), rabbit polyclonal anti-AKT, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology), goat polyclonal anti-SET, rabbit polyclonal anti-pAKT^{Thr308}, rabbit polyclonal anti-pERK1/2$^{Thr202/Tyr204}$ (Santa Cruz Biotechnology), rabbit polyclonal anti-PPP2R2A (Abcam), goat polyclonal anti-PPP2R5E (Novus Biologicals), rabbit polyclonal anti-CIP2A, and mouse monoclonal anti-bactin (Sigma). Proteins

were detected with the appropriate secondary antibodies conjugated to alkaline phospatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems). Densitometric analyses were performed using the Scion Image software (Scion Corporation). In the case of the experiments involving p-PP2A, p-PP2A-C/PP2A-C ratios of CRC cell lines were normalized to normal control. For CRC patient samples, ratios were normalized to paired normal colonic mucosa in each CRC case.

Cell viability assay

Cell proliferation was measured in triplicate wells by the MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). For that we thaw the Cell Titer reagent from approximately 90 minutes at room temperature. Next, we pipet 20µl of the reagent into each well of the 96-well assay containing the samples in 100 µl culture medium and incubate the plate at 37ºC for 2 hours in a humidified, 5% CO_2 atmosphere. Finally, we record the absorbance at 490 nm using a 96-well plate reader.

PP2A phosphatase activity assays

Protein extracts were isolated from cell lysates or fresh frozen CRC patient samples (5–10 tissue sections of 15 µm/each) using Trizol Reagent (Invitrogen). PP2A assays were performed with protein extracts (CRC cell lines: 50 µg; CRC patient samples: 100 µg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore). Briefly, PP2A was immunoprecipitated using 4 µg of PP2A antibody and 25 µl Protein A agarose slurry, both supplied by the kit. After 2 hours of incubation in constant rocking, samples were washed 3 times with TBS 1X followed by one additional wash with a ser/thr assay buffer also provided by the kit. Next, 60 µl of a diluted phosphopeptide at 750 µM and 40 µl of ser/thr assay buffer were added, and the mix was incubated for 5 min at 30°C in a shaking incubator, and then 25 µl of the mix was transferred into each well of a 96-well plate. Each measurement was performed in triplicates. 100 µl of Malachite Green Detection Solution was added, and the mix was incubated for 15 min at room temperature. Absorbance at 650 nm was used to calculate the amount of phosphate released (pmol) using a standard curve (0–2000 pmol).

Analysis of caspase activation

Quantification of caspase-3/7 activities was carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5 10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase-3/7 substrate containing a tetrapeptide Asp–Glu–Val–Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a Fluostar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity are expressed as fold-change of luminescence.

Colonosphere formation

We generated colonosphere-derived cells from DLD-1, SW480 and HT-29 cells using 6-well ultra-low attachment plates (Corning) and 10,000 cells per well. Cells were grown in serum-free DMEM/F12 supplemented with GlutMAXTM-I (Gibco) 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/ml human FGF (Sigma) and 50 ng/ml EGF (Sigma). After 7 days, plates were analyzed for colonosphere formation.

Colony-forming assay (Soft-agar)

Experiments were performed in 6-well plates coated with 3 mL of 0.6% soft agarose (Sigma) in medium. A total of 1 x 10^3 cells were suspended in 0.3% agarose in medium and plated in triplicates over the precoated wells. Fresh medium was supplied once a week. After 15 days, colonies were stained with MTT (M-5655, Sigma) for 4 hours at 37ºC. Then, colonies were fixed by adding dimethyl sulfoxide (DMSO) overnight at 37ºC. Colony numbers were determined from triplicates and three independent experiments were carried out for each condition and cell line.

Luciferase assay

Luciferase assays were done using the Dual Glo Luciferase Assay System (Promega). SW480 cells were transfected with 20 nM of pre-miR-199b (Ambion) and a pmiR-Glo construct empty or including the SET 3´UTR with the wild type or mutated miR-199b seed region. Firefly luciferase activities were normalized to Renilla luciferase activities.

Ex vivo models

Tissue slices, which were not needed for diagnostic purposes, from primary colorectal tumors larger than 1.5 cm of maximum diameter and normal adjacent colon mucosa were obtained from surgical specimens in patients newly diagnosed for adenocarcinoma. The samples were processed in sterile conditions immediately after surgical resection. Two slices (2 cm^3) for tumor and normal mucosa were used. One of the tumor and mucosa slides (designated as control samples) were put into 2 ml of RPMI plus 10% FBS and another additional tumor and mucosa slices (designated as treated samples) were put on the same culture medium plus FTY720 at 10 µM. Incubation was performed in 24- well plates at 37ºC in a constant atmosphere of 5% CO2 for 24 hours. At 24 hours, the specimens were fixed in 10% neutral-buffered formalin for 16 hours at room temperature and embedded in paraffin under vacuum conditions. These specimens were assayed for molecular markers by IHC analysis.

Clinical validation. Patient samples

Fresh-frozen samples of 35 colorectal cancer patients with paired tumor and normal colonic mucosa

The study comprised fresh-frozen samples of 35 patients with CRC obtained from surgical specimens provided by the Biobank of Fundacion Jimenez Díaz. Paired normal mucosa obtained from each patient was used as control. A pathologist confirmed that primary tumor tissues used in this work contained more than 70% tumoral components. More details of the cohort are detail in the tables of articles 1, 3 and 4 of the present doctorate thesis (see results: scientific publications)

FFPE tumor samples of 145 patients without metastatic colorectal cancer

Those patients were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data, and no metastatic disease. More details of the cohort are explained in article 4 of the present doctorate thesis (see results: scientific publications)

FFPE tumor samples of 250 patients with metastatic colorectal cancer

Those patients were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data, and metastatic disease. More details of the cohort are explained in articles 2, 4 and 5 of the present doctorate thesis (see results: scientific publications)

FFPE tumor samples of 92 patients with a histologic diagnosis of locally advanced rectal adenocarcinoma

Those patients were treated with neoadjuvant CRT between 2007 and 2013 at the University Hospital Fundación Jiménez Díaz and they were retrospectively selected for this study. All patients had an accurate preoperative loco regional staging accomplished with magnetic resonance (MRI) of the pelvis and/or transrectal ultrasound (TRUS). A full body computed tomography scan (FBCT) to exclude stage IV disease was performed in all participants. The patients received rule-based chemoradiotherapy regimens based on 5-FU, whether capecitabine and underwent surgery after 6 to 8 weeks after CRT completion. More details of the cohort are explained in articles 6 and 7 of the present doctorate thesis (see results: scientific publications).

Anatomopathological and molecular pathological analysis

TNM

Staging was classified using the 7th American Joint Committee on Cancer (AJCC) staging system for CRC.[221]

KRAS

KRAS mutational status was determined using the Cobas KRAS Mutation Test Kit (Roche Molecular Diagnostics).

Tissue microarrys

TMA (tissue microarrays) were constructed. Representative areas of each tumor were carefully selected and three tissue cores (1 mm diameter) were obtained using a TMA workstation (T1000 Chemicon).

Immunohistochemistry

Tissue sections (3 mm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 minutes. Slides were incubated with same primary antibody against SET, PP2A or p-PP2A depending on the experiment for 60 minutes at room temperature, followed of appropriate anti-Ig horseradish peroxidase conjugated polymer (Flexþ; Dako). Sections were visualized with 3,3'-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with normal non-immunized rabbit immunoglobulins were used as negative controls. As positive control, a section of colorectal tumor with known expression of SET, PP2A or p-PP2A was used. The antibody sensitivity had been calculated in a range of crescent dilutions of primary antibody. Specificity was confirmed in a set of paired fresh-frozen

and FFPE samples were processed by Western blot analysis and IHC. Only the membrane of epithelial cells, but not stromal cells, was evaluated blinded to clinical data by two pathologists. A semiquantitative histoscore was calculated by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low%) x 1 + (medium%) x 2 (high%) x 3 and the results ranged from 0 to 300.

Pathological response

For the rectal cancer cohort, two independent pathologists who were blinded to patient outcome evaluated tumor regression grade according to the modified Ryan classification which categorize tumors in four levels of response: complete response, moderate response, minimal response, and poor response. Complete response refers to no viable cancer cells and score 0; moderate response refers to single cells or small groups of cancer cells and score 1; minimal response refers to residual cancer outgrown by fibrosis and score 2; poor response refers to minimal or no tumor kill with extensive residual cancer and scores 3. According to clinical guidelines, all regression grades were assessed in the primary tumor.

Statistical analysis

We performed a descriptive analysis of each cohort clinico-pathological baseline characteristics as well as a descriptive analysis of the molecular alterations present at each cohort. Then, we proceed to correlate the molecular alterations with the clinical and pathological characteristics. For that, we applied the chi-square test (Fisher exact test) based on bimodal distribution of data to evaluate the correlation between the molecular alterations and the clinical and pathological variables.

The molecular alterations studies were analyzed as categoric variables after determining the optimal cut-off point of the continuous variables resulting from a ROC curve.

For the survival analysis we applied the Kaplan-Meyer method and the definition of our endpoints was as follow:

- DFS: the time from surgery (in case of the LARC cohort) or the time from the metastatic diagnosis (in case of the mCRC cohort) until recurrence, appearance of a secondary tumor, or death.
- OS: the time from the date of diagnosis (in case of the LARC cohort) or the time from the mCRC diagnosis (in case of the mCRC cohort) to the date of last follow-up or death.

Moreover, a univariate Cox analysis was performed to evaluate the potential prognostic value of each molecular alteration and next, taking into consideration significant parameters in univariate analysis we did multivariate analyses to evaluate the potential independent prognostic or predictive value of our molecular alterations or biomarkers.

To calculate the sensitivity, specificity, positive predictive value and negative predictive value we did a 2x2 crosstabulation (contingency table) representing our biomarkers findings with the true positive value (TP), the false positive value (FP), the false negative (FN) value and the true negative (TN) value. Next we calculated as follow: Sensitivity=TP/(TP+FN), Specificity= TN/(FP+TN), Positive predictive value= TP/(TP+FP), Negative predictive value=TN/(FN+TN)

All statistical analyses were performed using SPSS 20 for windows (SPSS Inc.) and a P value less than 0.05 was considered statistically significant.

As mentioned before all the manuscripts were elaborated in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines.[219]

RESULTS: SCIENTIFIC PUBLICATIONS

Article 1: PP2A Inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential.[1]

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

Molecular Cancer Therapeutics

PP2A Inhibition Is a Common Event in Colorectal Cancer and Its Restoration Using FTY720 Shows Promising Therapeutic Potential

Ion Cristóbal, Rebeca Manso, Raúl Rincón, et al.

Mol Cancer Ther 2014;13:938-947. Published OnlineFirst January 21, 2014.

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

PP2A Inhibition Is a Common Event in Colorectal Cancer and Its Restoration Using FTY720 Shows Promising Therapeutic Potential

Ion Cristóbal[1], Rebeca Manso[2], Raúl Rincón[2], Cristina Caramés[1], Clara Senin[3], Aurea Borrero[1], Javier Martínez-Useros[1], María Rodriguez[1], Sandra Zazo[2], Oscar Aguilera[1], Juan Madoz-Gúrpide[2], Federico Rojo[2], and Jesús García-Foncillas[1]

Abstract

Protein phosphatase 2A (PP2A) is a tumor suppressor that regulates many signaling pathways crucial for cell transformation. In fact, decreased activity of PP2A has been reported as a recurrent alteration in many types of cancer. Here, we show that PP2A is frequently inactivated in patients with colorectal cancer, indicating that PP2A represents a potential therapeutic target for this disease. We identified overexpression of the endogenous PP2A inhibitors SET and CIP2A, and downregulation of regulatory PP2A such as PPP2R2A and PPP2R5E, as contributing mechanisms to PP2A inhibition in colorectal cancer. Moreover, we observed that its restoration using FTY720 impairs proliferation and clonogenic potential of colorectal cancer cells, induces caspase-dependent apoptosis, and affects AKT and extracellular signal-regulated kinase-1/2 activation status. Interestingly, treatment with FTY720 showed an additive effect with 5-fluorouracil, SN-38, and oxaliplatin, drugs used in standard chemotherapy in patients with colorectal cancer. These results suggest that PP2A activity is commonly decreased in colorectal cancer cells, and that the use of PP2A activators, such as FTY720, might represent a potential novel therapeutic strategy in colorectal cancer. *Mol Cancer Ther; 13(4); 938–47. ©2014 AACR.*

Introduction

Colorectal cancer is a high prevalent neoplasia characterized by a progressive accumulation of genetic and epigenetic abnormalities that lead to cancer progression. Protein phosphatase 2A (PP2A) is a tumor suppressor that regulates many signaling pathways (1–3), and its loss of function has been associated with cell transformation (4–5). PP2A is not a single entity but a heterotrimeric complex consisting of a scaffold PP2A-A subunit, a catalytic PP2A-C subunit, and a regulatory PP2A-B subunit that determines both the specific substrate and the localization of the holoenzyme. Two isoforms have been described for PP2A-A and PP2A-C, and at least 26 isoforms for PP2A-B (6–7). Therefore, PP2A can form a high number of different complexes that interact with many different substrates (5).

It has been reported that transformed cells use different mechanisms to inhibit PP2A, including alterations in any of the PP2A subunits, and also the overexpression of specific endogenous inhibitors (4, 8–9). Despite PP2A has been described as a novel therapeutic target in several tumor models (10–12), the importance and potential value of PP2A as a drugable tumor suppressor in colorectal cancer remains mostly underexplored. Interestingly, there are some evidences that would suggest a relevant role of PP2A deregulation in colorectal cancer development. Alterations affecting *PPP2R1B* have been reported in colorectal cancer, impairing the interaction between PP2A-B and PP2A-C therefore inactivating PP2A (13–16). Furthermore, the tumor suppressor activity of PP2A seems to modulate the sensitivity of colorectal cancer cells to different treatments such as rapamycin (17), sphingadienes (18), or antiangiogenesis therapies (19). Therefore, we hypothesized that PP2A would be deregulated and could represent a novel targeted therapeutic strategy in colorectal cancer.

In this study, we show that PP2A activity is reduced in colorectal cancer, and overexpression of the endogenous PP2A inhibitors, SET or CIP2A, was identified as possible mechanisms of PP2A inhibition in colorectal cancer. Interestingly, FTY720 treatment increased PP2A activity,

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-13-0150

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

affecting proliferation and clonogenic potential of colorectal cancer cells. In addition, FTY720-induced PP2A activation led to increased apoptosis and changes in the phosphorylation status of AKT and extracellular signal-regulated kinase (ERK)-1/2. Of importance, our data provide strong evidences that PP2A activation could be a promising therapeutic target in combination with drugs used in standard chemotherapy, such as 5-fluorouracil (5-FU), SN-38, or oxaliplatin (LOHP).

Materials and Methods

Cell cultures

The human colorectal cancer cell lines RKO (ATCC CRL-2577), SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38), LoVo (ATCC CCL-229), and SW620 (ATCC CCL-227) were purchased from American Type Culture Collection (ATCC) and were not cultured for more than 2 months. No authentication was done by the authors. Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% FBS and were grown at 37ºC in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/mL) and streptomycin (0.1 mg/mL). Cells were treated with the following reagents: 5-FU (1 μmol/L; Sigma), SN-38 (50 nmol/L; Sigma), LOHP (1 μmol/L; Sigma), FTY720 (10 μmol/L; Calbiochem), and okadaic acid (2.5 nmol/L; Calbiochem).

Patient samples

The study comprised fresh-frozen samples of 21 patients with colorectal cancer obtained from surgical specimens provided by the Biobank of Fundación Jiménez Díaz (Madrid, Spain). Paired normal mucosa obtained from each patient was used as control. A pathologist confirmed that primary tumor tissues used in this work contained more than 70% tumoral components. The Ethical Committee and Institutional Review Board approved the project.

Direct nucleotide sequencing

Reverse transcription reactions were performed using SuperScript III (Invitrogen). The amplified PCR products were purified from agarose gel after electrophoresis using Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and sequencing reactions were carried out by duplicates using forward and reverse primers to confirm the results obtained and performed by the company Secugen, S.L. Results were analyzed using Chromas Version 2.4.1 and ClustalW2 bioinformatic tools.

Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following manufacturer's indications, clarified (12,000 × *g*, 15 minutes, 4ºC), denatured, and subjected to SDS-PAGE and Western blot analysis. Antibodies used were mouse monoclonal anti-PP2A (clone 1D6, Upstate Inc.), rabbit polyclonal anti-PP2A (FL-309; Santa Cruz Biotechnology), rabbit monoclonal anti-PP2AY307 (Epitomics), rabbit polyclonal anti-AKT, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology), goat polyclonal anti-SET, rabbit polyclonal anti-pAKT^{Thr308}, rabbit polyclonal anti-pERK1/2$^{Thr202/Tyr204}$ (Santa Cruz Biotechnology), rabbit polyclonal anti-PPP2R2A (Abcam), goat polyclonal anti-PPP2R5E (Novus Biologicals), rabbit polyclonal anti-CIP2A, and mouse monoclonal anti-βactin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phospatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems).

Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer's indications.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described (20).

Analysis of caspase activation

Quantification of caspase-3/7 activities in untreated or FTY720-treated colorectal cancer cells were carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5×10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase-3/7 substrate containing a tetrapeptide Asp-Glu-Val-Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal were measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity in FTY720-treated cells compared with untreated cells are expressed as fold-change in luminescence.

Colony-forming assay

Experiments were performed in 6-well plates coated with 3 mL of 0.6% soft agarose (Sigma) in medium. A total of 1×10^3 cells were suspended in 0.3% agarose in medium and plated in triplicates over the precoated wells. Fresh medium was supplied once a week. After 15 days, colonies were stained with MTT (M-5655, Sigma) for 4 hours at 37°C. Then, colonies were fixed by adding dimethyl sulfoxide (DMSO) overnight at 37°C. Colony numbers were determined from triplicates and three independent experiments were carried out for each condition and cell line.

Ex vivo models

Tissue slices, which were not needed for diagnostic purposes, from primary colorectal tumors larger than 1.5 cm of maximum diameter and normal adjacent colon mucosa were obtained from surgical specimens in patients newly diagnosed for adenocarcinoma. The samples were processed in sterile conditions immediately

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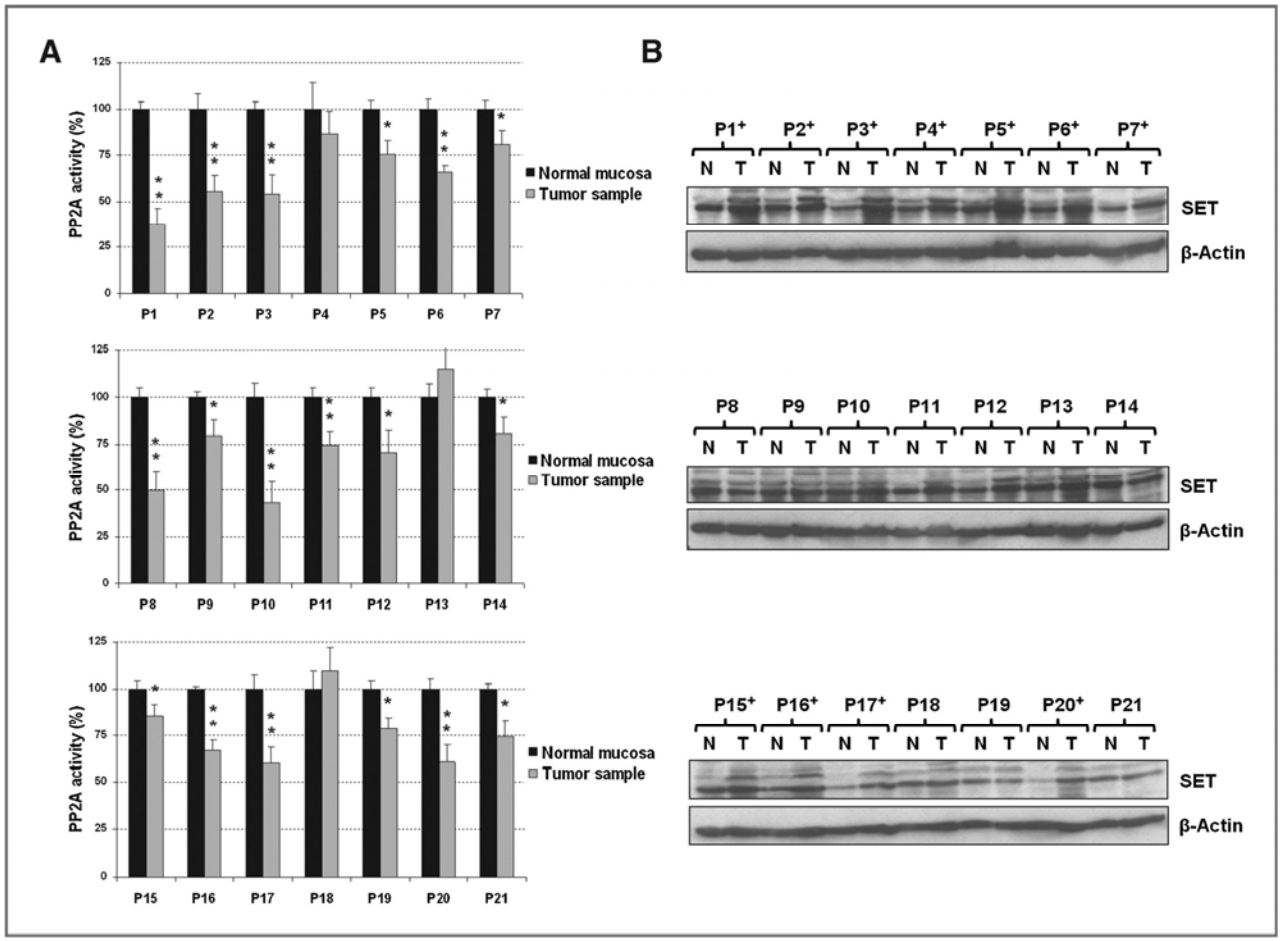

Figure 1. PP2A shows reduced activity in patients with colorectal cancer. A, quantification of PP2A activity in patients with colorectal cancer with normal mucosa and tumor samples. *, $P < 0.05$; **, $P < 0.01$. B, Western blot analysis of SET expression in colorectal cancer patient samples; +, SET overexpression; N, normal mucosa; T, tumor sample.

after surgical resection. Two slices (2 cm^3) for tumor and normal mucosa were used for the present study. One of the tumor and mucosa slides (designated as control samples) were put into culture medium, and another additional tumor and mucosa slices (designated as treated samples) were put on the same culture medium plus FTY720 at 10 μmol/L. Incubation was performed in 24-well plates at 37°C in a constant atmosphere of 5% CO2 for 24 hours. At 24 hours, the specimens were fixed in 10% neutral-buffered formalin for 16 hours at room temperature and embedded in paraffin under vacuum conditions. These specimens were assayed for molecular markers as described in the immunohistochemical (IHC) section.

Immunohistochemistry

Tissue sections (3 μm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 minutes. Slides were incubated with primary antibodies for 60 minutes at room temperature, followed of appropriate anti-Immunoglobulin horseradish peroxidase-conjugated polymer (Flex+, Dako). Sections were visualized with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with nonimmunized serum were used as negative controls.

Results

Reduced PP2A activity and SET deregulation are common events in colorectal cancer

To evaluate the importance of PP2A in colorectal cancer, we analyzed PP2A activation status in a series of 21 patients diagnosed of colorectal cancer. Patient characteristics are indicated in Supplementary Table S1. Interestingly, we observed a significant reduction of PP2A activity in 18 out of the 21 samples analyzed (Fig. 1A and Supplementary Fig. S1 and Supplementary Table S2). Because the 1D6 antibody against PP2A has been previously reported to recognize the demethylated fraction of PP2A (21–22), we performed these experiments with a second antibody against full-length PP2A (FL-309), and similar results were observed in all cases except for P4 who was

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

positive for PP2A inhibition only with the FL-309 antibody (Supplementary Figs. S2 and S3). To investigate the possible causes of PP2A inhibition, we sequenced *PPP2R1B* in the 21 patients with colorectal cancer. Information about the primers used for amplifying *PPP2R1B* cDNA segments is indicated in the Supplementary Table S3. Two silent heterozygous alterations were found: GAC (Asp) to GAT (Asp) at codon 391 in the patient 12 (P12), and TAT (Tyr) to TAC (Tyr) at codon 72 in patient 14 (P14; Supplementary Fig. S4). However, no missense mutations were identified in our cohort. In addition, we analyzed by Western blot analysis the expression of the PP2A endogenous inhibitor SET in these patients with colorectal cancer. We found SET overexpressed in 13 out the 21 samples analyzed (Fig. 1B and Supplementary Table S2). Taken together, these results would indicate that PP2A inactivation is a common event in colorectal cancer and that deregulation of the endogenous PP2A inhibitor SET would be a key contributing mechanism to PP2A inactivation in colorectal cancer.

CIP2A is frequently overexpressed and correlates with c-MYC levels in colorectal cancer

The fact that some of the patients included in our series showed reduced PP2A activity without displaying SET deregulation prompted us to study the presence of alternative mechanisms to inhibit PP2A in colorectal cancer. We studied the levels of CIP2A, another endogenous PP2A inhibitor recently reported as deregulated in colorectal cancer (23). Interestingly, we found CIP2A overexpressed in 15 out of 21 cases analyzed (Fig. 2 and Supplementary Table S2). CIP2A acts inhibiting PP2A-mediated c-MYC dephosphorylation and proteolytic degradation (24). Furthermore, CIP2A has been proposed as a key c-Myc regulator in colorectal cancer (25) and, in concordance with this point, we observed a good correlation between CIP2A and c-MYC levels in our series (Fig. 2). Therefore, these observations would suggest that CIP2A overexpression is another contributing mechanism to inhibit PP2A in colorectal cancer. To further investigate other possible mechanisms of PP2A inhibition in colorectal cancer, we analyzed by Western blot analysis, the expression of PPP2R2A and PPP2R5E. Interestingly, we found PPP2R2A downregulated in nine out of the 21 patients with colorectal cancer, and PPP2R5E downregulation in three out of 21 cases (Supplementary Table S2 and Supplementary Fig. S5). These observations would suggest that downregulation of regulatory PP2A subunits such as PPP2R2A and PPP2R5E could be involved in the PP2A inhibition observed in colorectal cancer.

PP2A activation by FTY720 reduces cell viability in colorectal cancer cells

We next performed functional analyses in a series of seven colorectal cancer cell lines to clarify the biologic relevance of PP2A deregulation in colorectal cancer. We first analyzed the PP2A status and the expression levels of SET and CIP2A in our panel of colorectal cancer cell lines

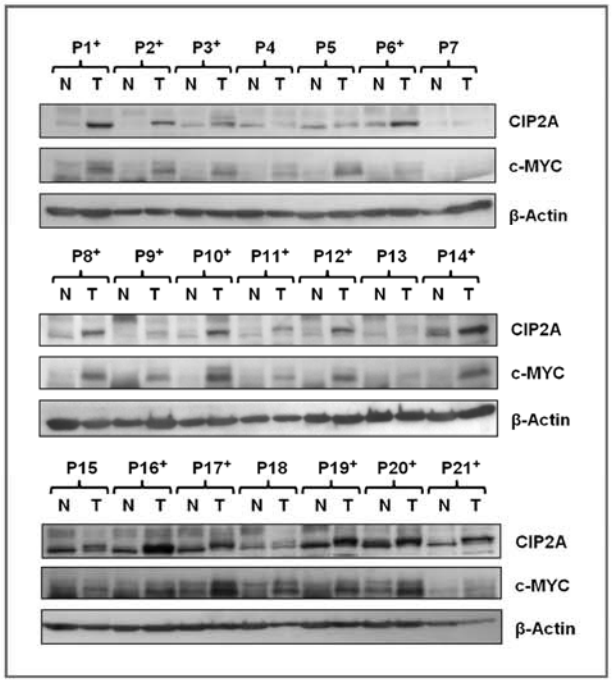

Figure 2. CIP2A is overexpressed in colorectal cancer and correlates with c-MYC levels. Western blot analysis of CIP2A expression in colorectal cancer patient samples; $^{+}$, CIP2A overexpression; N, normal mucosa; T, tumor sample.

observing that all of them showed PP2A inhibition together with overexpression of both SET and CIP2A in comparison with normal controls (Supplementary Fig. S6). To assess whether increased PP2A activity affects cell proliferation of colorectal cancer cells, RKO and LoVo cell lines were treated with the PP2A activator FTY720 or vehicle (DMSO). Phosphatase assays to quantify PP2A activity levels confirmed that FTY720 treatment leads to PP2A activation (Fig. 3A and Supplementary Fig. S7). In addition, we pretreated RKO and LoVo cells with the PP2A inhibitor okadaic acid for 90 minutes, followed by incubation with FTY720 or vehicle for 24 hours. FTY720-induced PP2A activity in RKO and LoVo cells was inhibited by okadaic acid (Fig. 3A). Western blot analysis showed that similar levels of PP2Ac protein were immunoprecipitated in the PP2A phosphatase assays (Fig. 3B), suggesting that differences observed in PP2A activity are not due to changes in PP2Ac expression levels. We next analyzed the effect of PP2A activation on cell growth using MTS assay. We observed a decreased proliferation in FTY720-treated RKO and LoVo cells compared with vehicle-treated cells (Fig. 3C). Similar results were obtained with the SW480, HT-29, and DLD-1 cell lines (Supplementary Fig. S8). In addition, we observed that the impaired proliferation induced by FTY720 was partially rescued by the treatment with okadaic acid used at a concentration that inhibits PP2A but no other phosphatases (Fig. 3C and Supplementary Fig. S8; ref. 26). Moreover, the images obtained after treatment with FTY720, using an optical microscope, were in concordance with the

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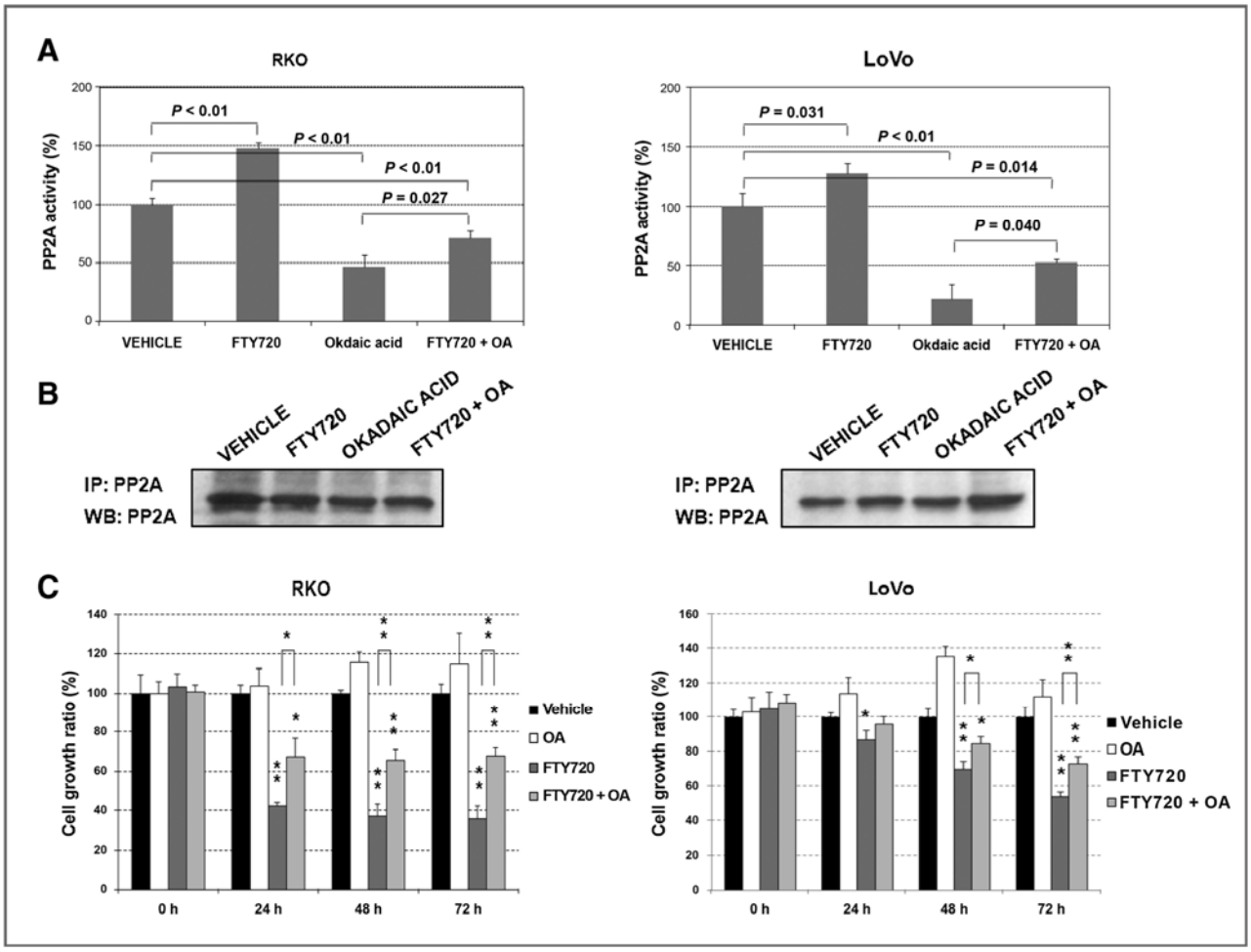

Figure 3. FTY720 treatment induces an impaired proliferation via PP2A activation. A, treatment with okadaic acid (OA) inhibits the FTY720-induced PP2A activity in RKO and LoVo cells. B, levels of immunoprecipitated PP2A (1D6 antibody) from the RKO and LoVo lysates used in the phosphatase assays. C, the impaired cell viability induced by FTY720 is partially rescued by the treatment with okadaic acid. *, $P < 0.05$; **, $P < 0.01$.

MTS results (Supplementary Fig. S9). Altogether, these results show that PP2A activation by FTY720 treatment induces toxicity in colorectal cancer cells.

FTY720 leads to an impaired clonogenic potential of colorectal cancer cells that is dependent on PP2A activation

To further confirm the importance of FTY720-modulating colorectal cancer cell proliferation, we determined the effects on colony-forming ability of FTY720 treatment in colorectal cancer cells. Interestingly, FTY720-treated RKO and LoVo cells formed a markedly reduced number of colonies than controls (Fig. 4). In concordance with the results obtained in the proliferation assays, we observed that the impaired clonogenic ability induced by FTY720 was partially rescued by the treatment with okadaic acid, indicating that this effect is at least partially promoted by PP2A activation. Similar results were also obtained with the HT-29 and DLD-1 cell lines (Supplementary Fig. S10). These results would suggest a potential therapeutic value for FTY720 treatment through PP2A activation in colorectal cancer cells.

FTY720 induces apoptosis in colorectal cancer cells

To further investigate the biologic effect of the FTY720-induced PP2A activation in colorectal cancer, RKO cells were treated with FTY720, and then we assessed apoptosis measuring activity levels of caspase-3 and -7. Vehicle-treated cells were used as controls. Consistent with its ability to increase PP2A activity and suppress cell growth, FTY720 showed a caspase-dependent proapoptotic effect, increasing caspase activity levels almost 7-fold in FTY720-treated RKO cells compared with vehicle-treated cells (Fig. 5A). In addition, caspase activity in FTY720-treated cells was markedly reduced when cells were pretreated with okadaic acid. These results were confirmed in the LoVo cell line (Fig. 5A).

Molecular effects of PP2A activation in colorectal cancer cells after FTY720 treatment

We next analyzed by Western blot analysis whether the FTY720 treatment had any effect in the phosphorylation status of previously described PP2A targets. Consistent with previous reports about the effects of

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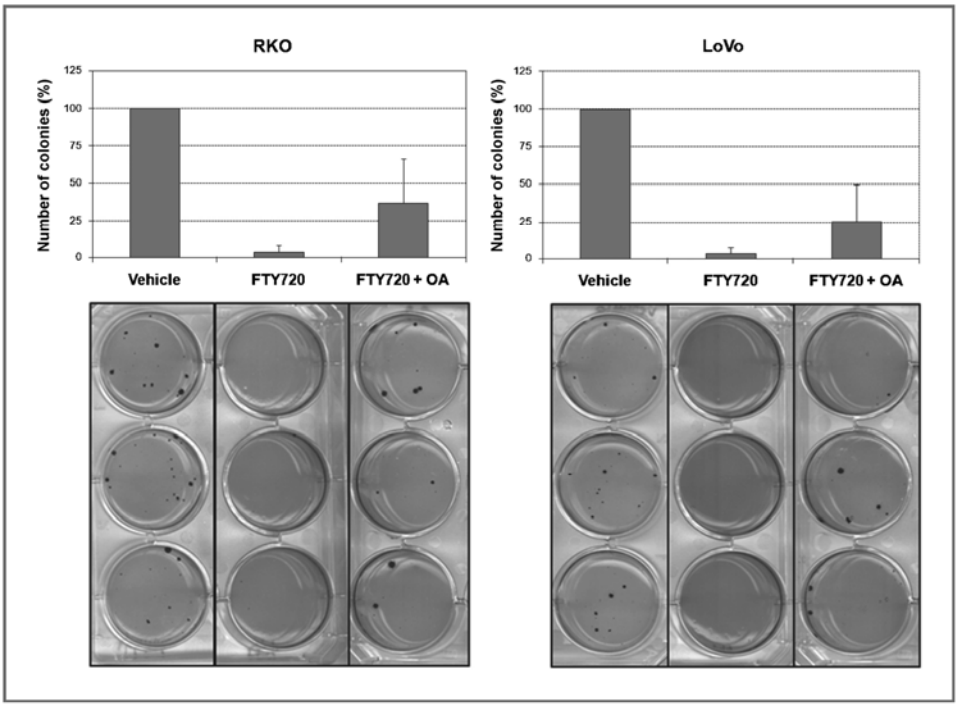

Figure 4. Colony-forming ability of untreated, FTY720-treated, and FTY720/okadaic acid (OA)-treated RKO and LoVo cells. Colony-forming assay showing effects of FTY720 alone or in combination with okadaic acid in RKO and LoVo cells.

PP2A activation in other tumor models (27), FTY720 treatment decreased phosphorylation (activity) of the PP2A targets AKT and ERK1/2 without affecting their expression levels (Fig. 5B). Moreover, okadaic acid treatment rescued AKT and ERK1/2 phosphorylation in FTY720-treated RKO cells. Similar results were observed in LoVo (Fig. 5B), HT-29, and SW480 cells (Supplementary Fig. S11). Analysis in the HT-29 cell line showed decreased phosphorylation only for ERK1/2 (Supplementary Fig. S11). Moreover, we observed that phosphorylation on tyrosine 307 of PP2Ac was not affected in cells treated with FTY720 compared with cells treated with vehicle (DMSO; Supplementary Fig. S11). Altogether, these results would indicate that the molecular mechanism of action of FTY720 involves the inhibition of AKT and ERK1/2 signaling.

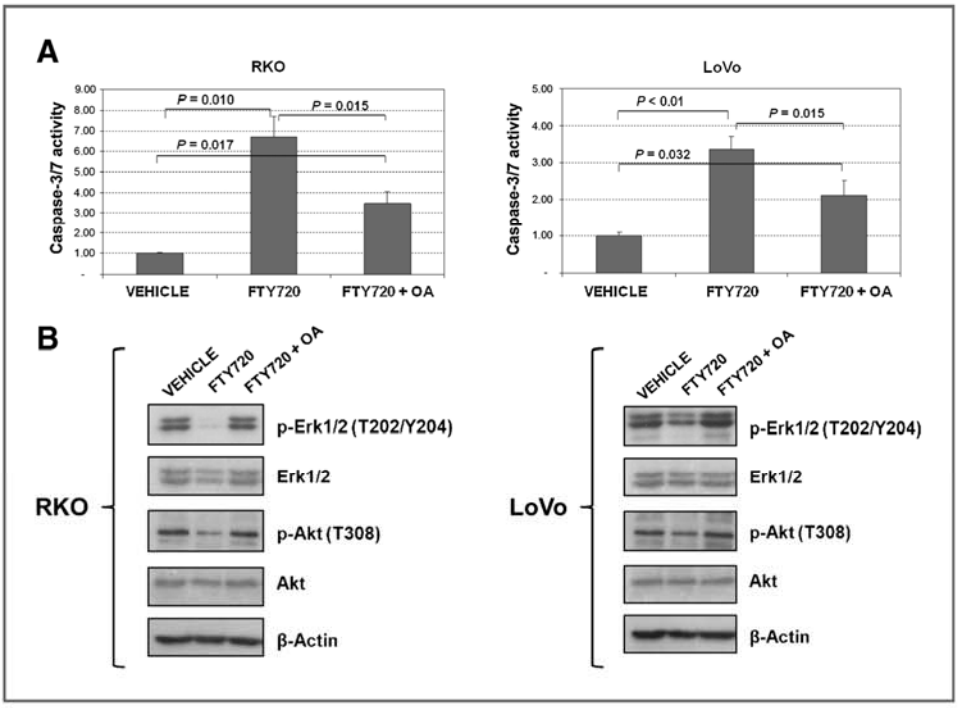

Figure 5. FTY720 induces caspase-dependent apoptosis together with changes in the phosphorylation status of PP2A targets. A, caspase-3/7 assays in RKO and LoVo cells untreated (DMSO), and FTY720-treated alone or in combination with okadaic acid (OA). B, Western blot analysis showing AKT and ERK1/2 phosphorylation and expression after FTY720 treatment in RKO and LoVo cells.

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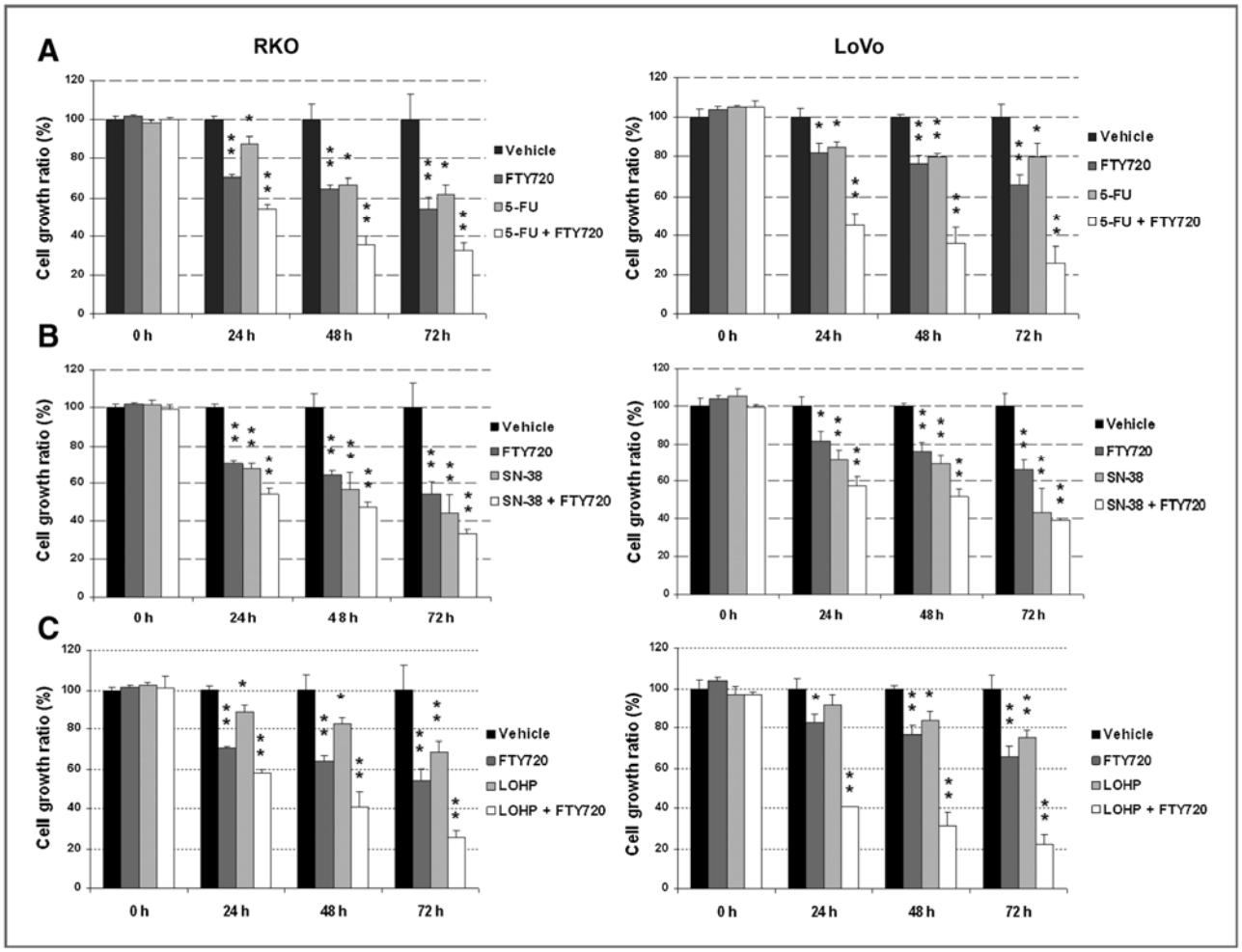

Figure 6. FTY720 treatment shows an additive effect with 5-FU, SN-38, and LOHP treatments in colorectal cancer (CRC) cells. MTS assays showing the effect of FTY720 and 5-FU (A), SN-38 (B) or LOHP (C) alone or in combination in RKO and LoVo cells. Cells treated with vehicle (DMSO) were used as controls. *, $P < 0.05$; **, $P < 0.01$.

We next analyzed PP2A, CIP2A, and SET levels after treatment with FTY720 alone or in combination with okadaic acid in RKO and LoVo cell lines. Interestingly, Western blot analysis showed that CIP2A decreased after FTY720 treatment and CIP2A levels were restored when cells where treated with FTY720 in combination with okadaic acid. No changes were observed in SET or PP2A expression. As expected, c-MYC levels decreased in correlation with CIP2A (Supplementary Fig. S12). Altogether, these results would describe a novel mechanism of action of FTY720 through CIP2A and c-MYC deregulation.

FTY720 potentiates antitumor activity of 5-fluorouracil, SN-38, and oxaliplatin in colorectal cancer cells

To assess the effect of a combination between standard induction chemotherapy drugs used in colorectal cancer and FTY720, we treated colorectal cancer cells with either 5-FU, SN-38, or LOHP, alone or in combination with FTY720. Of importance, we observed that FTY720 enhanced the antitumor effects mediated by 5-FU (Fig. 6A), SN-38 (Fig. 6B), and LOHP (Fig. 6C) treatments in the RKO and LoVo cell lines. These data were also confirmed in DLD-1 (Supplementary Fig. S13) and HT-29 cells (Supplementary Fig. S14). Therefore, these results showed that FTY720 treatment has an additive antitumor effect when combined with either 5-FU, SN-38, or LOHP in colorectal cancer cells.

FTY720 shows therapeutic effects in human colorectal cancer *ex vivo* models

To further investigate the potential therapeutic effects of FTY720 in colorectal cancer and assess cytotoxicity of this drug in normal colon cells, we performed human colorectal cancer *ex vivo* models using five primary colorectal cancer tumors that were obtained from surgical specimens of patients newly diagnosed for adenocarcinoma. IHC analysis comparing samples of normal colonic mucosa and colorectal cancer from the same patient

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

showed that FTY720 treatment induces an increased apoptosis (cleaved caspase-3) together with decreased proliferation (Ki-67) in tumor samples, whereas no significant changes were observed in control samples (Supplementary Fig. S15). These observations would support the observations made *in vitro* and indicate that cytotoxic effects of FTY720 are markedly reduced in normal colon cells.

Discussion

PP2A is a human tumor suppressor that regulates the activity of several signaling proteins critical for malignant cell behavior. We report here that PP2A is frequently inactivated in colorectal cancer cell lines and patient samples. In addition, we demonstrate that FTY720-induced PP2A activation leads to cell growth inhibition, impaired clonogenic potential, caspase-dependent apoptosis, and changes in the activation status of downstream targets such as AKT and ERK1/2. Importantly, FTY720 treatment shows an additive effect with the chemotherapy reagents 5-FU, SN-38, and LOHP, suggesting that treatment with PP2A activators could be a novel therapeutic option in colorectal cancer in combination with standard chemotherapy.

To evaluate the importance of PP2A deregulation in colorectal cancer, we quantified PP2A activity levels in 21 patients with colorectal cancer (paired normal mucosa and tumor samples), observing a reduced PP2A activity in 19 out of 21 colorectal cancer patient samples (Fig. 1 and Supplementary Fig. S2 and Supplementary Table S2). PP2A assays were carried out with two different antibodies against PP2A (1D6 and FL-309) that showed similar results in all cases except in P4 (PP2A inhibition only with FL-309). Although the FL-309 antibody recognizes the full-length PP2A, the 1D6 antibody has been reported to preferentially recognize the demethylated fraction of PP2A (21–22), and this could be an explanation for the discrepancy observed in this case P4 between the PP2A assays performed with these antibodies (Supplementary Table S2). To determine the molecular mechanisms responsible of this PP2A inhibition in colorectal cancer, we sequenced *PPP2R1B* but no missense mutations were found in our cohort. These results would confirm previous works reporting that cancer-associated mutations affecting the *PPP2R1B* gene are rare events in colorectal cancer (13–16). We then decided to assess the expression levels of the PP2A endogenous inhibitors SET and CIP2A, observing deregulation of SET in 13 out of 21 cases, and overexpression of CIP2A in 15 out of 21 colorectal cancer patient samples (Figs. 1B and 2), which indicates that these alterations are recurrent events in colorectal cancer. Very interestingly, we observed no alterations in SET and/or CIP2A in our colorectal cancer cases without PP2A inhibition. Moreover, of 19 patients with reduced PP2A activity, six cases showed CIP2A overexpression, four SET overexpression, and nine both SET and CIP2A deregulation (Supplementary Table S2). Data of PP2A activity, SET expression, and CIP2A expression status have been indicated for each patient in the Supplementary Table S2. CIP2A is a protein that inhibits PP2A-mediated c-MYC dephosphorylation and proteolytic degradation then increasing c-MYC protein stability (24). In addition, it has been reported that CIP2A overexpression is associated with c-MYC expression in colorectal cancer (25), and we showed a correlation between CIP2A and c-MYC levels (Fig. 2) that would confirm the previous observations made by Böckelman and colleagues. Thoroughly, these results would indicate that overexpression of SET and CIP2A are contributing mechanisms that could cooperate simultaneously to inhibit PP2A in colorectal cancer.

Together with the overexpression of endogenous PP2A inhibitors and cancer-associated mutations affecting structural PP2A subunits, downregulation of regulatory PP2A subunits has been described as a mechanism to inactivate PP2A in cancer cells (4, 9). Thus, we analyzed PPP2R2A and PPP2R5E, two regulatory PP2A subunits recently implicated in cancer (28–29) observing downregulation of these subunits in, respectively, nine and three out of the 21 colorectal cancer cases (Supplementary Table S2 and Supplementary Fig. S5). These alterations could contribute to explain some of the differences observed in the PP2A activity levels of the patients with colorectal cancer. However, PP2A constitutes a large family of serine/threonine phosphatases, and in this work, we have only tested some of the potential mechanisms to inhibit PP2A. Although the catalytic and the scaffold subunits have two different variants, four unrelated families of PP2A regulatory subunits have been identified, including at least 26 different alternative transcripts and splice forms (5). Thus, PP2A has the ability to form a high number of different complexes with an also high number of potential alterations that could contribute to PP2A inhibition affecting any of these PP2A subunits. In fact, the cancerous cell shows a wide variety of molecular strategies to inhibit PP2A, including hyperphosphorylation or downregulation of its catalytic subunit, mutations or downregulation affecting any of the scaffold or regulatory subunits, and the overexpression of endogenous PP2A inhibitors such as SET or CIP2A, but also others such as SETBP1 or SRC (20, 30). Therefore, further investigation would be necessary to clarify the particular importance of each mechanism and to identify other potential mechanisms involved in the PP2A inactivation observed in colorectal cancer.

To assess the potential therapeutic value of PP2A activation, we used FTY720 to treat colorectal cancer cells. FTY720 is an immunosuppressor and U.S. Food and Drug Administration (FDA)-approved drug for multiple sclerosis treatment. Interestingly, FTY720 has shown antitumor properties in several cancers. We considered this drug to treat colorectal cancer because together with its FDA approbation, Nagaoka and colleagues studied the effect of FTY720 phosphorylation in breast and colon cancer, observing an anticancer activity against two colorectal cancer cell lines (HCT-116 and SW620; ref. 31). In addition, the pharmacologic activation of PP2A using

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

FTY720 has been proposed as a therapeutic alternative for future treatments in patients with some leukemias (27, 32). Interestingly, it has been recently reported that FTY720 targets SET and then mediates tumor suppression via PP2A activation in lung cancer (33). Our results show that PP2A inhibition plays an important role in colorectal cancer transformation because the pharmacologic activation of PP2A *in vitro* and *ex vivo* using FTY720 reverses some of the malignant features of the colorectal cancer cells, whereas cytotoxic effects seem to be markedly impaired in normal colon cells (Figs. 3–5 and Supplementary Fig. S15). Moreover, we observed that the molecular mechanism by which FTY720 is acting involves the inhibition of the AKT and ERK1/2 (Fig. 5B), both PP2A targets, suggesting that this effect occurs via PP2A activation. In fact, we showed PP2A activation after FTY720 treatment but, unexpectedly, we did not observe any change in the PP2A phosphorylation levels (Supplementary Fig. S11), indicating that FTY720 activates PP2A by a mechanism alternative to the tyrosine-307 dephosphorylation. Interestingly, we observed that FTY720 led to decreased CIP2A levels (Supplementary Fig. S12), which indicates that in addition to its recently reported blockade of SET (33), FTY720 could be acting decreasing CIP2A expression. Indeed, these two events are in concordance with the PP2A activation observed after the FTY720 treatment because SET and CIP2A are two endogenous PP2A inhibitors. Furthermore, our results also showed that FTY720 exhibits additive antitumor effects with reagents used in standard chemotherapy such as 5-FU, SN-38 (active metabolite of irinotecan), and LOHP (Fig. 6), suggesting that FTY720 is a good candidate for future trials in combination with standard chemotherapy reagents in patients with colorectal cancer.

In conclusion, we report that functional inactivation of PP2A is a common event in colorectal cancer. In addition, we show that functional loss of PP2A activity occurs through contributing mechanisms such as overexpression of the PP2A endogenous inhibitors, SET and CIP2A, and downregulation of the regulatory PP2A subunits, PPP2R2A and PPP2R5E. Moreover, the potent antitumor effects observed after restoration of PP2A activity with FTY720 indicates that PP2A inhibition is an alteration with high relevance in colorectal cancer pathogenesis. Finally, the fact that FTY720 treatment has shown an additive effect to chemotherapy drugs highlights PP2A as a molecular target with a potential value in therapies combined with PP2A activators in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: I. Cristobal, C. Carames, O. Aguilera, J. Madoz-Gurpide, F. Rojo, J. Garcia-Foncillas
Development of methodology: I. Cristobal, R. Manso, R. Rincon, C. Carames, C. Senin, A. Borrero, J. Martinez-Useros
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Rincon, C. Carames, C. Senin, S. Zazo, F. Rojo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Cristobal, R. Manso, R. Rincon, C. Carames, C. Senin, F. Rojo, J. Garcia-Foncillas
Writing, review, and/or revision of the manuscript: I. Cristobal, C. Carames, O. Aguilera, J. Madoz-Gurpide, F. Rojo, J. Garcia-Foncillas
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Manso, R. Rincon, C. Senin, M. Rodriguez, F. Rojo
Study supervision: I. Cristobal, J. Garcia-Foncillas

Acknowledgments

The authors thank Monica Martinez and Carmen Cañadas for technical assistance.

Grant Support

This work was supported by Biobank of Fundación Jiménez Díaz (FJD Biobank) grants from Instituto de Salud Carlos III FEDER; RD09/0076/00101. R. Manso was supported by Fundacion Conchita Rabago de Jiménez Diaz.

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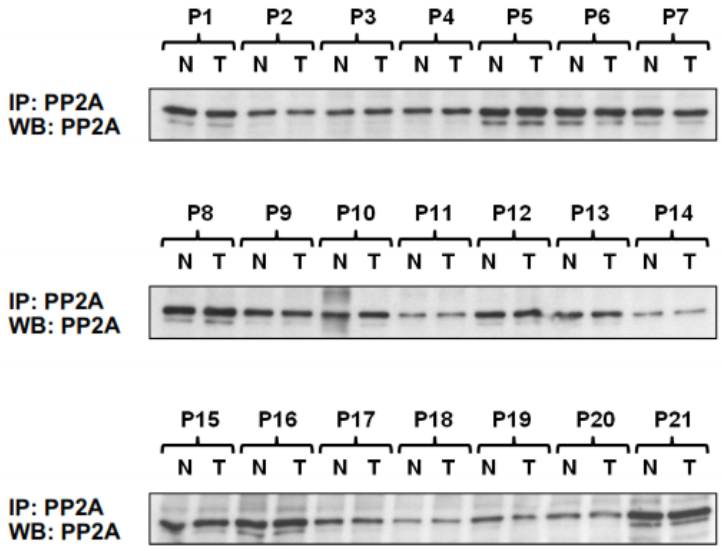
Received February 27, 2013; revised December 3, 2013; accepted January 9, 2014; published OnlineFirst January 21, 2014.

References

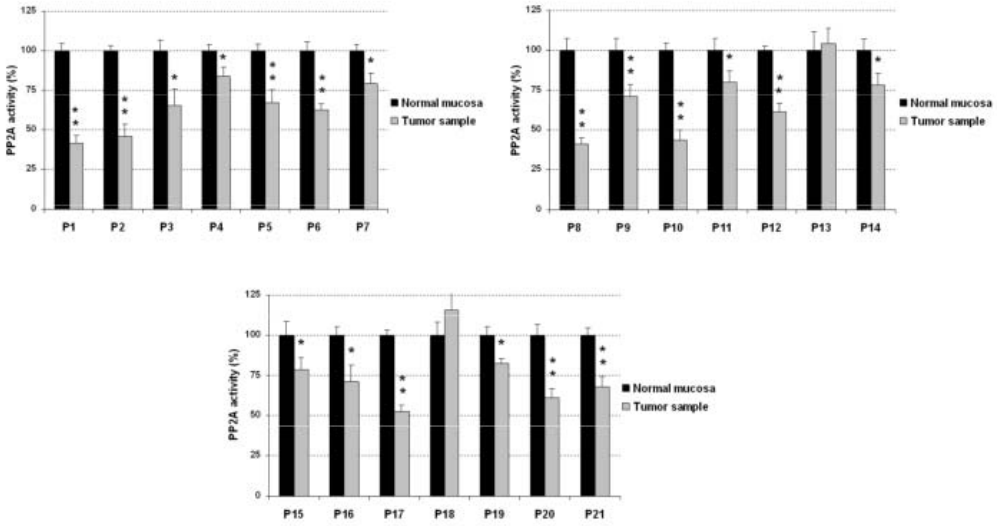
1. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 2001;353:417–39.
2. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. Curr Opin Genet Dev 2005;15:34–41.
3. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007;130:21–4.
4. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med 2008;14:152–160.
5. Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. Biochim Biophys Acta 2009;1795:1–15.
6. Zhou J, Pham HT, Ruediger R, Walter G. Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. Biochem J 2003;369:387–98.
7. Arino J, Woon CW, Brautigan DL, Miller TBJr, Johnson GL. Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. Proc Natl Acad Sci U S A 1988;85:4252–56.
8. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science 1992;257:1261–4.
9. Cristobal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD. PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. Leukemia 2011;25:606–14.
10. Perrotti D, Neviani P. Protein phosphatase 2A (PP2A), a drugable tumor suppressor in Ph1(+) leukemias. Cancer Metastasis Rev 2008;27:159–68.
11. Voronkov M, Braithwaite SP, Stock JB. Phosphoprotein phosphatase 2A: a novel druggable target for Alzheimer's disease. Future Med Chem 2011;3:821–33.
12. Kalev P, Sablina AA. Protein phosphatase 2A as a potential target for anticancer therapy. Anticancer Agents Med Chem 2011;11:38–46.
13. Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J, et al. Alterations of the PPP2R1B gene in human lung and colon cancer. Science 1998;282:284–7.

Published OnlineFirst January 21, 2014; DOI: 10.1158/1535-7163.MCT-13-0150

14. Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. Gut 2000;47:268–71.
15. Ruediger R, Pham HT, Walter G. Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. Oncogene 2001;20:1892–9.
16. Tamaki M, Goi T, Hirono Y, Katayama K, Yamaguchi A. PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. Oncol Rep 2004;11:655–9.
17. Tan J, Lee PL, Li Z, Lim YC, Hooi SC, et al. B55β-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. Cancer Cell 2010;18:459–71.
18. Kumar A, Pandurangan AK, Lu F, Fyrst H, Zhang M, Byun HS, et al. Chemopreventive sphingadienes downregulate Wnt signaling via a PP2A/Akt/GSK3β pathway in colon cancer. Carcinogenesis 2012;33:1726–35.
19. Lin SP, Lee YT, Yang SH, Miller SA, Chiou SH, Hung MC, et al. Colon cancer stem cells resist antiangiogenesis therapy-induced apoptosis. Cancer Lett 2013;328:226–34.
20. Cristobal I, Blanco FJ, Garcia-Orti L, Marcotegui N, Vicente C, Rifon J, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. Blood 2010;115:615–625.
21. Wei H, Ashby DG, Moreno CS, Ogris E, Yeong FM, Corbett AH , et al. Carboxymethylation of the PP2A catalytic subunit in Saccharomyces cerevisiae is required for efficient interaction with the B-type subunits Cdc55p and Rts1p. J Biol Chem 2001;276:1570–1577.
22. MacKay KB, Tu Y, Clarke SG. Circumventing embryonic lethality with Lcmt1 deficiency: generation of hypomorphic Lcmt1 mice with reduced protein phosphatase 2A methyltransferase expression and defects in insulin signaling. PLoS One 2013;8:e65967.
23. Teng HW, Yang SH, Lin JK, Chen WS, Lin TC, Jiang JK , et al. CIP2A is a predictor of poor prognosis in colon cancer. J Gastrointest Surg 2012;16:1037–47.
24. Junttila MR, Westermarck J. Mechanisms of MYC stabilization in human malignancies. Cell Cycle 2008;7:592–6.
25. Böckelman C, Koskensalo S, Hagström J, Lundin M, Ristimäki A, Haglund C. CIP2A overexpression is associated with c-Myc expression in colorectal cancer. Cancer Biol Ther 2012;13:289–95.
26. Saydam G, Aydin HH, Sahin F, Selvi N, Oktem G, Terzioglu E , et al. Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. Leuk Res 2003;27:709–717.
27. Neviani P, Santhanam R, Oaks JJ, Eiring AM, Notari M, Blaser BW , et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. J Clin Invest 2007;117:2408–21.
28. Kalev P, Simicek M, Vazquez I, Munck S, Chen L, Soin T , et al. Loss of PPP2R2A inhibits homologous recombination DNA repair and predicts tumor sensitivity to PARP inhibition. Cancer Res 2012;72:6414–24.
29. Cristobal I, Cirauqui C, Castello-Cross R, Garcia-Orti L, Calasanz MJ, Odero MD. Downregulation of PPP2R5E is a common event in acute myeloid leukemia that affects the oncogenic potential of leukemic cells. Haematologica 2013;98:e103–104.
30. Barisic S, Schmidt C, Walczak H, Kulms D. Tyrosine phosphatase inhibition triggers sustained canonical serine-dependent NFkappaB activation via Src-dependent blockade of PP2A. Biochem Pharmacol 2010;80:439–447.
31. Nagaoka Y, Otsuki K, Fujita T, Uesato S. Effects of Phosphorylation of Immunomodulatory Agent FTY720 (Fingolimod) on Antiproliferative Activity against Breast and Colon Cancer Cells. Biol Pharm Bull 2008;31:1177–81.
32. Liu Q, Zhao X, Frissora F, Ma Y, Santhanam R, Jarjoura D , et al. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. Blood 2008;111:275–284.
33. Saddoughi SA, Gencer S, Peterson YK, Ward KE, Mukhopadhyay A, Oaks J , et al. Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumour suppression via activation of PP2A-RIPK1-dependent necroptosis. EMBO Mol Med 2013;5:105–21.



Supplementary Figure 1



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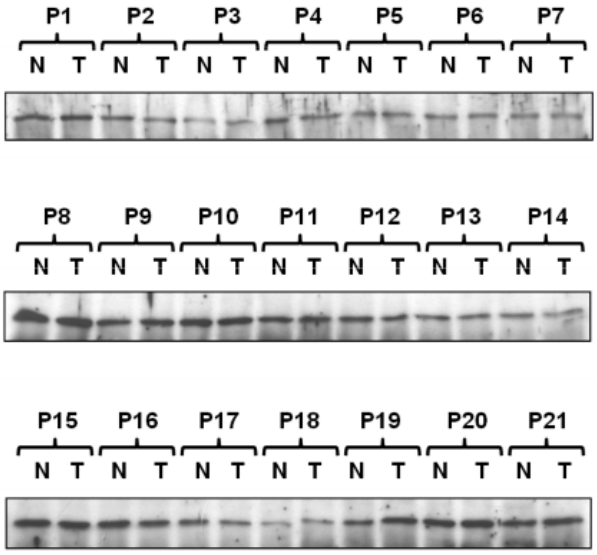

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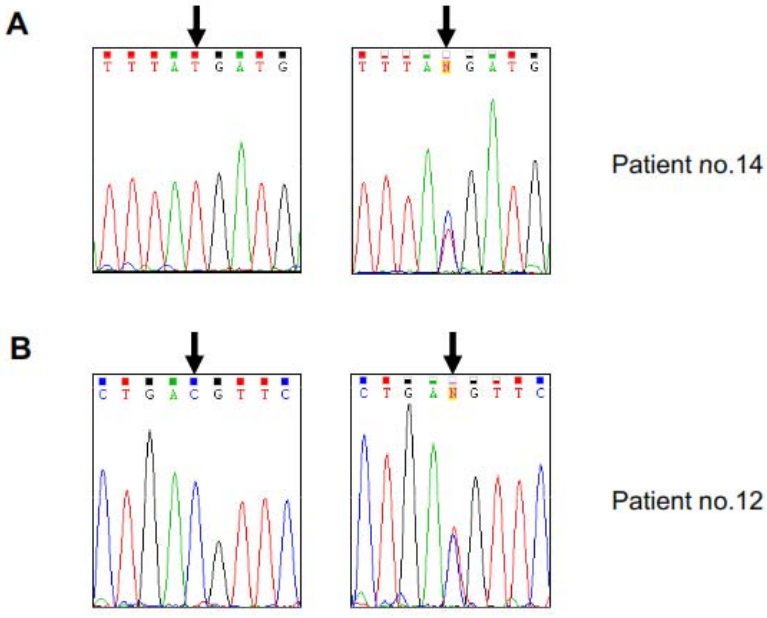
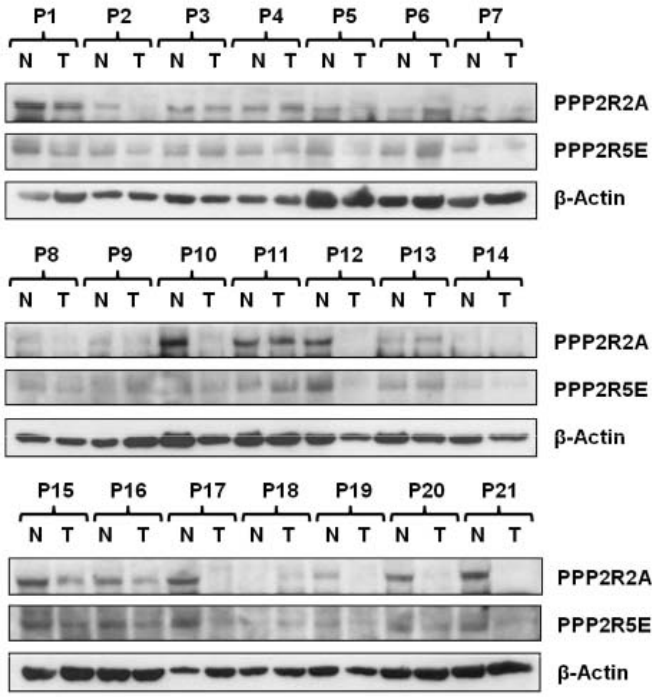
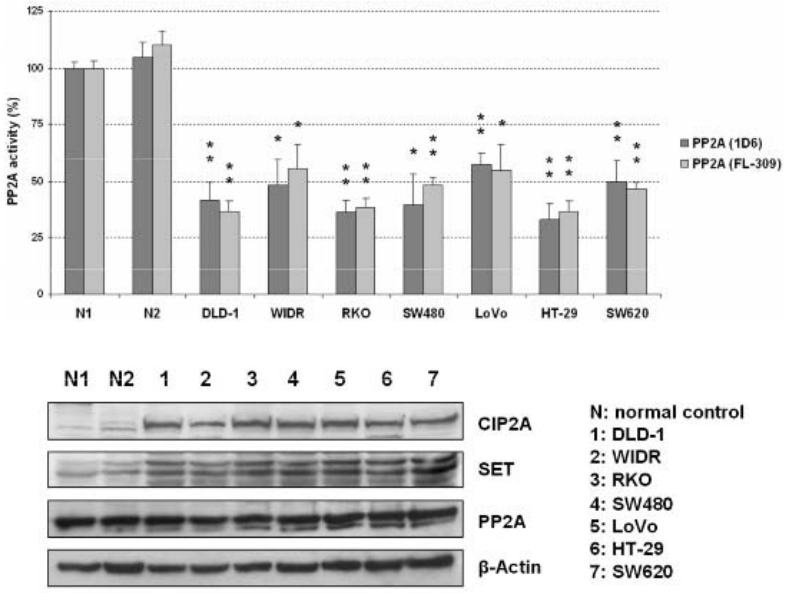


Supplementary Figure 4



Supplementary Figure 5



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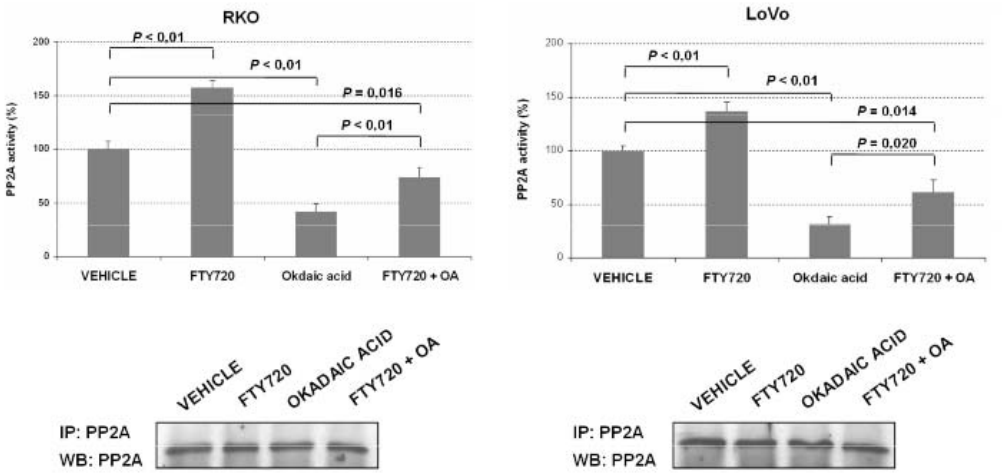
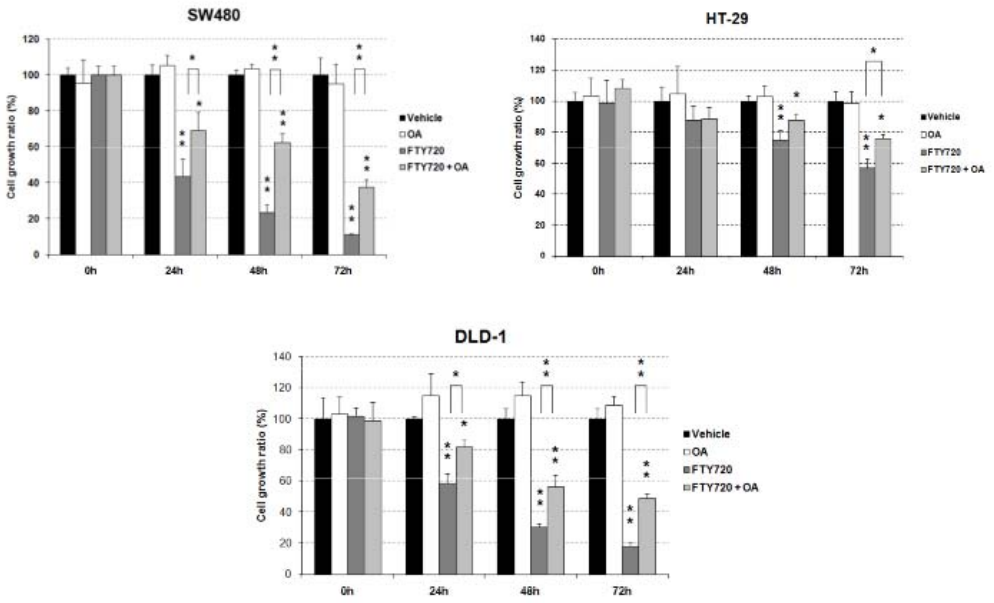
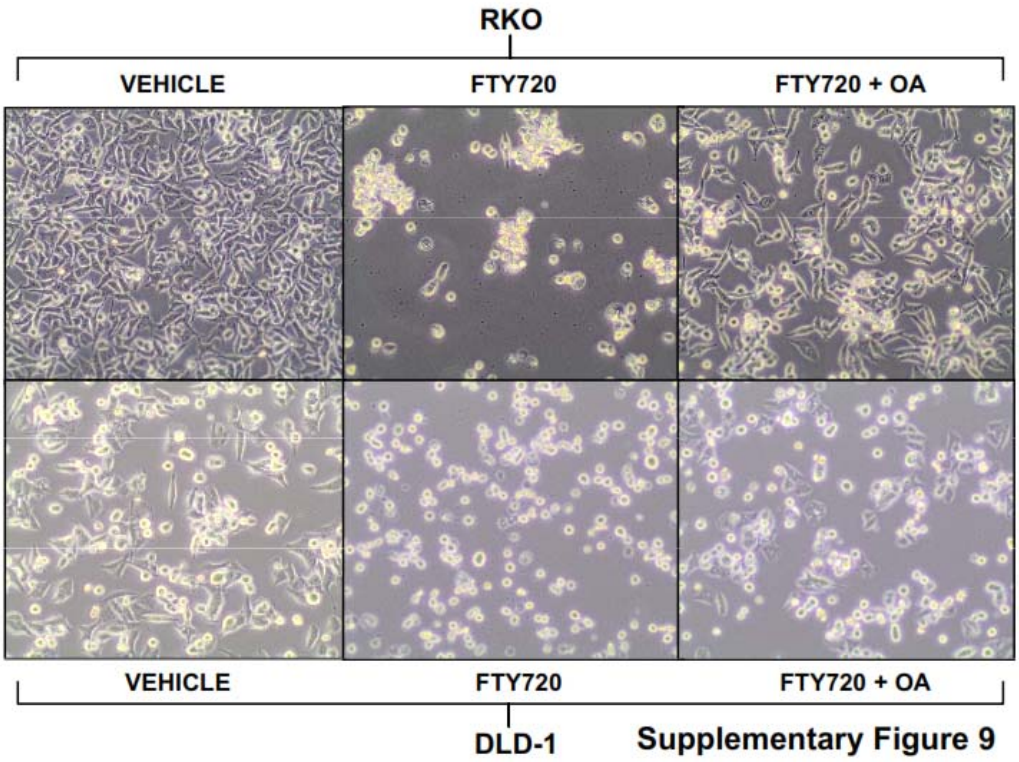


Supplementary Figure 7



Supplementary Figure 8



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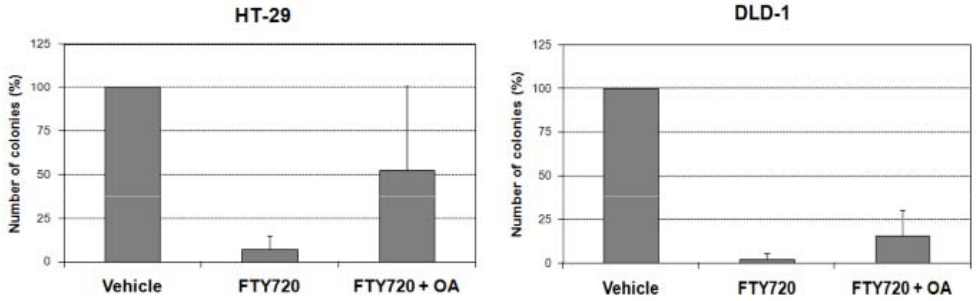
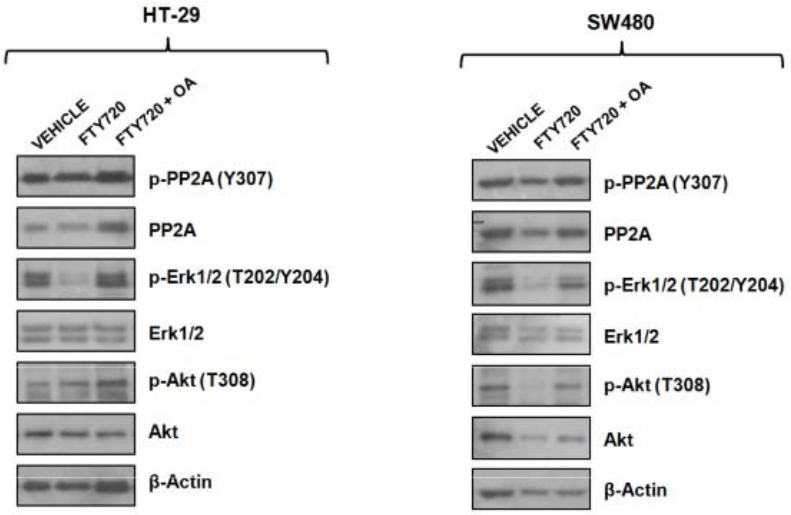
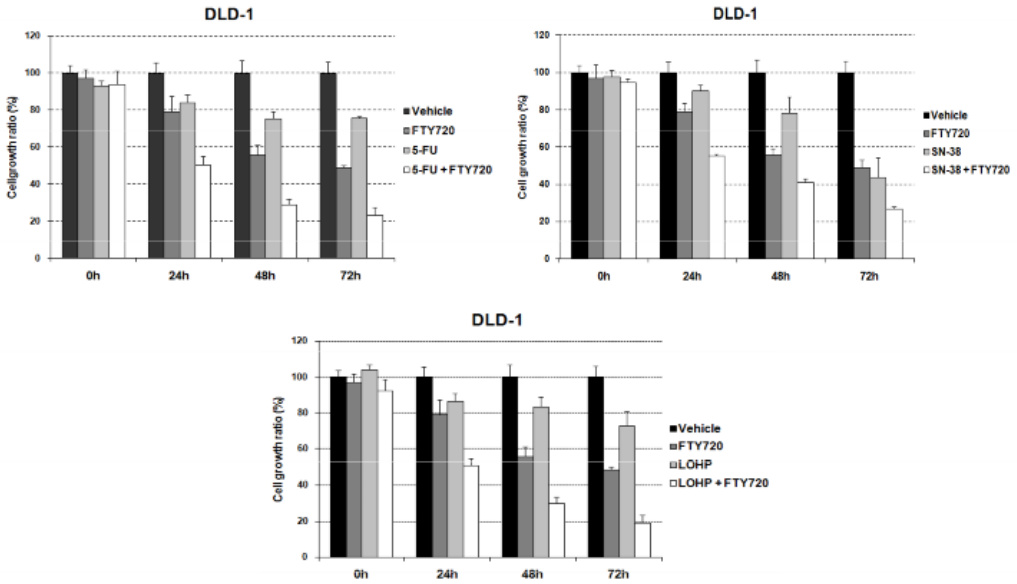


Supplementary Figure 10

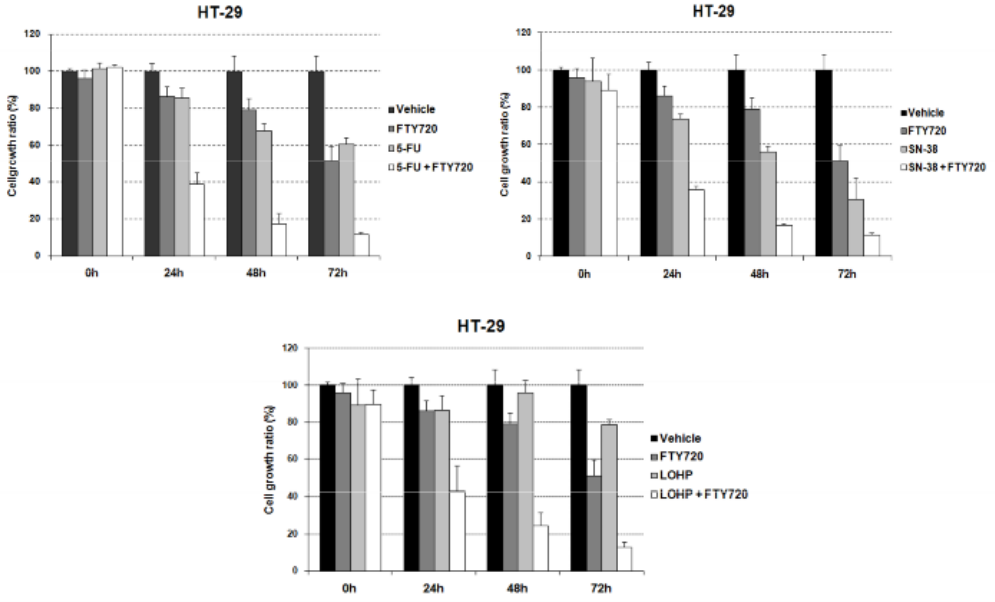


Supplementary Figure 11

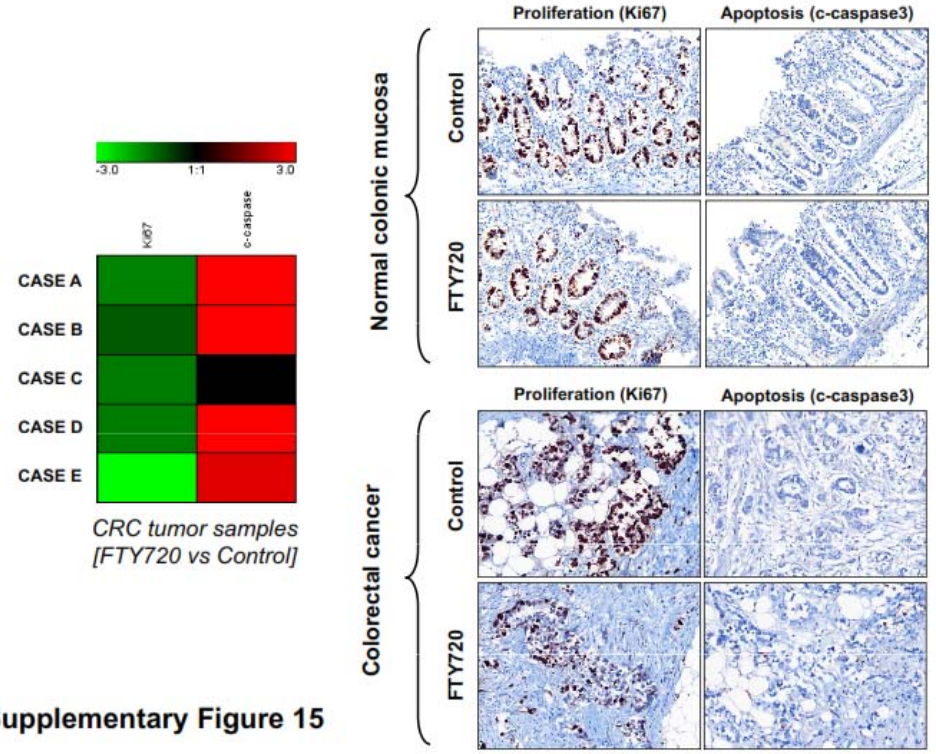
Supplementary Figure 12



Supplementary Figure 13



Supplementary Figure 14



Supplementary Figure 15

Article 2: p-PP2A determines poor outcome in patients with metastatic colorectal cancer.[2]

FULL PAPER

BJC

British Journal of Cancer (2014) 111, 756–762 | doi: 10.1038/bjc.2014.376

Keywords: p-PP2A; metastatic colorectal cancer; prognosis; therapy

Phosphorylated protein phosphatase 2A determines poor outcome in patients with metastatic colorectal cancer

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Background: Protein phosphatase 2A (PP2A) is a tumour suppressor frequently inactivated in human cancer and its tyrosine-307 phosphorylation has been reported as a molecular inhibitory mechanism.

Methods: Expression of phosphorylated PP2A (p-PP2A) was evaluated in 250 metastatic colorectal cancer (CRC) patients. Chi-square, Kaplan–Meier and Cox analyses were used to determine correlations with clinical and molecular parameters and impact on clinical outcomes.

Results: High p-PP2A levels were found in 17.2% cases and were associated with ECOG performance status ($P=0.001$) and presence of synchronous metastasis at diagnosis ($P=0.035$). This subgroup showed substantially worse overall survival (OS) (median OS, 6.0 *vs* 26.2 months, $P<0.001$) and progression-free survival (PFS) (median PFS, 3.8 *vs* 13.3 months, $P<0.001$). The prognostic impact of p-PP2A was particularly evident in patients aged <70 years ($P<0.001$). Multivariate analysis revealed that p-PP2A retained its prognostic impact for OS (hazard ratio 2.7; 95% confidence interval, 1.8–4.1; $P<0.001$) and PFS (hazard ratio 3.0; 95% confidence interval, 1.8–5.0; $P<0.001$).

Conclusions: Phosphorylated PP2A is an alteration that determines poor outcome in metastatic CRC and represents a novel potential therapeutic target in this disease, thus enabling to define a subgroup of patients who could benefit from future treatments based on PP2A activators.

Colorectal cancer (CRC) is the most common gastrointestinal cancer and its aetiology involves an interaction of genetic and epigenetic alterations with environmental factors such as diet components that contribute to cancer development. A correct balance between kinase and phosphatase activities is essential in maintaining cell homeostasis (Hunter, 1995). The presence of alterations affecting kinase activities has been shown to be recurrent in many cancers, and therapies based on kinase inhibitors have been developed in the previous years. Although the role of phosphatases remains underexplored in comparison, it is true that phosphatases like protein phosphatase 2A (PP2A) have progressively been considered as potential tumour suppressors.

Protein phosphatase 2A is a major serine/threonine phosphatase that consists of a heterotrimer that includes a catalytic, a scaffold, and a regulatory subunit involved in the specificity and localisation of the holoenzyme. Owing to the multiple existing isoforms and splicing variants for each PP2A subunit, especially in the case of the regulatory subunit, PP2A can form a wide variety of heterotrimeric complexes with distinct substrate specificities and they therefore have different biological functions (Arino *et al*, 1988; Zhou *et al*, 2003; Eichhorn *et al*, 2009). Of importance, PP2A plays a pivotal role in regulating many signalling pathways (Millward *et al*, 1999; Janssens and Goris, 2001; Janssens *et al*, 2005; Mumby, 2007) and its inactivation has been described as a common event in

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Received 27 January 2014; revised 20 May 2014; accepted 13 June 2014; published online 8 July 2014

the cancerous cell through several molecular strategies (Mumby, 2007; Westermarck and Hahn, 2008; Eichhorn *et al*, 2009). Interestingly, Chen *et al* (1992) demonstrated that *in vitro* phosphorylation of PP2A at Y307 by protein tyrosine kinases led to its inactivation. Moreover, phosphorylation of PP2A at Y307 has been described as a molecular PP2A-inactivating mechanism with relevance in chronic and acute leukaemias or Alzheimer's disease (Perrotti and Neviani, 2008; Cristobal *et al*, 2010, 2011; Xiong *et al*, 2013). Although PP2A has been proposed as a novel therapeutic target in several tumours (Perrotti and Neviani, 2008, 2013; Kalev and Sablina, 2011; Voronkov *et al*, 2011), the potential relevance of PP2A as a druggable tumour suppressor in CRC still needs to be clarified. However, several observations indicate that PP2A inhibition could be playing an important role in CRC development. Thus, the presence of reported PP2A-inactivating mutations affecting the scaffold PP2A subunit in CRC (Wang *et al*, 1998; Takagi *et al*, 2000; Ruediger *et al*, 2001; Tamaki *et al*, 2004), together with the fact that PP2A seems to modulate the sensitivity of CRC cells to different treatments (Tan *et al*, 2010; Kumar *et al*, 2012; Lin *et al*, 2012), prompted us to hypothesise that PP2A could represent a novel molecular target with relevance in CRC.

In this report, we studied p-PP2A in a cohort of 250 metastatic CRC patients, observing that high p-PP2A levels were associated with worse ECOG performance status and the presence of synchronous metastasis. Of importance, the high p-PP2A subgroup showed a markedly shorter overall survival (OS) and progression-free survival (PFS), with significance in both wild-type and mutated KRAS subgroups. Interestingly, multivariate analysis showed that p-PP2A has an independent prognostic value for OS and PFS in metastatic CRC.

MATERIALS AND METHODS

Patient samples. The study comprised consecutive formalin-fixed paraffin-embedded tumour samples of 250 patients with metastatic CRC that were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data and metastatic disease. Samples were surgical resection specimens from CRC tumours obtained from Fundacion Jimenez Diaz Biobank (BFJD, Madrid, Spain). TNM (tumour, Node, Metastases) staging was classified using the 7th American Joint Committee on Cancer staging system for CRC. Clinical data were collected from medical clinical records by oncologists (JG-F, CC). KRAS mutational status was determined using the Cobas KRAS Mutation Test kit (Roche Molecular Diagnostics, Branchburg, NJ, USA) following manufacturer's procedures. Tissue microarrays (TMAs) were constructed. Representative areas of each tumour were carefully selected and three tissue cores (1 mm diameter) were obtained using a TMA workstation (T1000 Chemicon, Temecula, CA, USA). Samples were taken anonymously. The ethics committee and institutional review board approved the project.

Immunohistochemistry. Tissue sections (3 μm) were placed on plus-charged glass slides. After deparaffinisation in xylene and graded alcohols, heat antigen retrieval was performed in pH 9 EDTA-based buffer (Dako, Glostrup, Denmark). Endogenous peroxidase was blocked by using 0.03% hydrogen peroxide for 5 min. Slides were incubated with the primary antibody against p-PP2A (rabbit monoclonal anti-p-PP2A Y307; Abcam, Cambridge, UK; ref.: ab32104) for 60 min at room temperature, followed by the appropriate anti-Ig horseradish peroxidase-conjugated polymer (Flex+, Dako). Sections were visualised with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with normal non-immunised rabbit immunoglobulin were used as negative controls. As positive control, a section of colorectal tumour with known expression of p-PP2A was used. p-PP2A antibody sensitivity (1 : 2000) had been calculated in a range of crescent dilutions of the primary antibody. Specificity was confirmed in a set of paired fresh frozen and formalin-fixed paraffin-embedded samples processed by western blot and IHC (Supplementary Figure 1). Moreover, additional controls to confirm the specificity of the antibody were performed and included PP2A silencing with specific siRNAs designed and synthesised by Dharmacon RNA Technologies (Dharmacon, Lafayette, CO, USA), *in vitro* dephosphorylation of SW480 and HT-29 cell lysates using calf intestinal phosphatase (CIP), and western blot analysis of CRC tumour tissues showing the entire membrane to demonstrate that any other major bands are recognised by the antibody (Supplementary Figure 2). Antibodies used were mouse monoclonal anti-PP2A (Upstate Inc., Lake Placid, NY, USA) and mouse monoclonal anti-βactin (Sigma, St Louis, MO, USA). Antigen preservation in tissues was confirmed by assaying sections from the same TMAs for expression of phospho-tyrosines by immunohistochemistry, using a monoclonal antibody to tyrosine-phosphorylated proteins (clone 4G10, 1 : 500, Millipore, Billerica, MA, USA). Those cases showing absent, irregular, or weak staining for the phospho-tyrosine assay were excluded from the analysis. Only the membrane of epithelial cells, but not stromal cells, was evaluated for p-PP2A expression in a manner blinded to clinical data by two pathologists (FR and SZ). A semiquantitative histoscore was calculated by estimating the percentage of tumour cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low %) × 1 + (medium %) × 2 + (high %) × 3 and the results ranged from 0 to 300.

Statistical analysis. Statistical analyses were performed using SPSS 20 for windows (SPSS Inc, Chicago, IL, USA). Overall survival was defined as the time from the date of metastatic diagnosis to the date of death from any cause or last follow-up. Progression-free survival was defined as the time from metastatic diagnosis until progression to first-line metastatic treatment or death. The Kaplan–Meier method and survival comparisons were done with the log-rank test if proportional hazard assumption was fulfilled and Breslow otherwise. The Cox proportional hazards model was adjusted by taking into consideration significant parameters in univariate analysis. A P-value <0.05 was considered statistically significant. Receiver operating curve was used to determine the optimal cutoff point based on progression end point for p-PP2A expression as previously described (Generali *et al*, 2009). This work was carried out in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies guidelines (McShane *et al*, 2005).

RESULTS

Prevalence of p-PP2A in metastatic CRC and its association with clinical and molecular parameters. To study the prevalence of p-PP2A and its potential clinical significance in CRC, we quantified the expression of p-PP2A by immunohistochemistry in a series of 250 patients with metastatic CRC, correlated the results obtained with clinical and molecular features, and studied the prognostic relevance of this aberration. Patient characteristics are presented in Supplementary Table 1. High p-PP2A expression was observed in 17.2% cases (43 out of 250). The prevalence of PP2A hyperphosphorylation was higher in women than in men (24.5% *vs* 12.8%, $P=0.018$). Moreover, we found high p-PP2A to be associated with worse ECOG performance status (35.7% *vs* 15.5%, $P=0.001$), and with the presence of synchronous metastasis (20.9% *vs* 11.3%, $P=0.035$). Association between p-PP2A and clinical and genetic parameters is shown in Table 1.

Table 1. Association between p-PP2A and clinical and genetic parameters in 250 patients with metastatic CRC

	No. of cases	No. p-PP2A − (%)	No. p-PP2A + (%)	*P*
p-PP2A	250	207 (82.8)	43 (17.2)	
Sex	250	207	43	**0.018**
Male	156	136 (87.2)	20 (12.8)	
Female	94	71 (75.5)	23 (24.5)	
Age	232	190	42	0.306
<70	116	98 (84.5)	18 (15.5)	
⩾70	116	92 (79.3)	24 (20.7)	
ECOG	224	184	40	**0.001**
0–2	182	157 (84.5)	25 (15.5)	
3–4	42	27 (64.3)	15 (35.7)	
Site of primary tumour	250	207	43	0.552
Cecum	24	17 (84.5)	7 (15.5)	
Right colon	37	31 (79.3)	6 (20.7)	
Transverse colon	10	7 (70)	3 (30)	
Left colon	21	18 (85.7)	3 (14.3)	
Sigma	73	61 (83.6)	12 (16.4)	
Rectum	85	73 (85.9)	12 (14.1)	
Synchronous metastasis	244	201	43	**0.035**
No	85	76 (89.4)	9 (10.6)	
Yes	159	125 (78.6)	34 (21.4)	
Number of metastatic sites	250	207	43	0.620
1–2	226	188 (83.2)	38 (16.8)	
>2	24	19 (79.2)	5 (20.8)	
Liver metastasis	244	201	43	0.294
No	79	68 (86.1)	11 (13.9)	
Yes	165	133 (80.6)	32 (19.4)	
Lung metastasis	244	201	43	0.323
No	166	134 (80.7)	32 (19.3)	
Yes	78	67 (85.9)	11 (14.1)	
Lymph metastasis	244	201	43	0.409
No	177	148 (83.6)	29 (16.4)	
Yes	67	53 (79.1)	14 (20.9)	
Peritoneal metastasis	244	201	43	0.846
No	196	161 (82.1)	35 (17.9)	
Yes	48	40 (83.3)	8 (16.7)	
MSI	240	189	41	0.774
No	226	187 (82.7)	39 (17.3)	
Yes	14	12 (85.7)	2 (14.3)	
KRAS mutations	246	203	43	0.873
No	140	116 (82.9)	24 (17.1)	
Yes	106	87 (72.1)	19 (17.9)	

Abbreviations: ECOG = Eastern Cooperative Oncology Group; MSI = magnetic source imaging; p-PP2A = protein phosphatase 2A. *P* values in bold font indicate differences statistically significant.

Clinical significance of p-PP2A in metastatic CRC. Clinical follow-up data were available for 243 cases, 149 men and 94 women, with a median age of 69.5 years (age range: 29–92). Median OS of the global cohort was 21.9 months (95% confidence interval, 17.2–26.6 months). We found that the subgroup of patients with high p-PP2A showed a substantially shorter OS (median OS, 6.0 *vs* 26.2 months, $P<0.001$) (Figure 1A) and PFS (median PFS, 3.8 *vs* 13.3 months, $P<0.001$) (Figure 1B). Interestingly, the prognostic impact of p-PP2A was particularly evident in the subgroup of patients aged <70 years (median OS, 6.2 *vs* 33.2 months, $P<0.001$; median PFS, 4.4 *vs* 16.4 months, $P<0.001$); however, significance was also achieved in the subgroup of elderly patients (median OS, 5.9 *vs* 15.2 months, $P=0.012$; median PFS, 3.8 *vs* 7.6 months, $P=0.020$) (Figure 2). To further investigate the

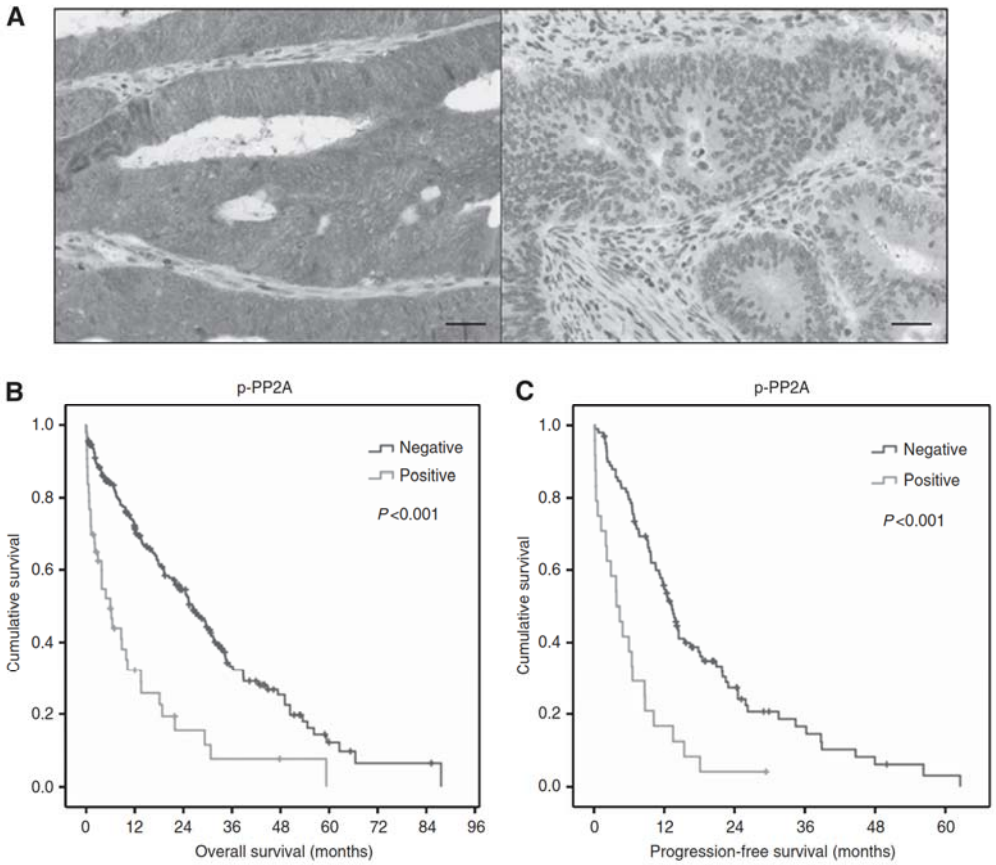


Figure 1. Clinical significance of p-PP2A in metastatic CRC: (**A**) Immunohistochemical detection of p-PP2A showing positive and negative staining. The line shows 25 μm. Magnification $\times$ 400; Kaplan–Meier analyses of overall survival (**B**) and progression-free survival (**C**) in a cohort of 243 patients with metastatic CRC.

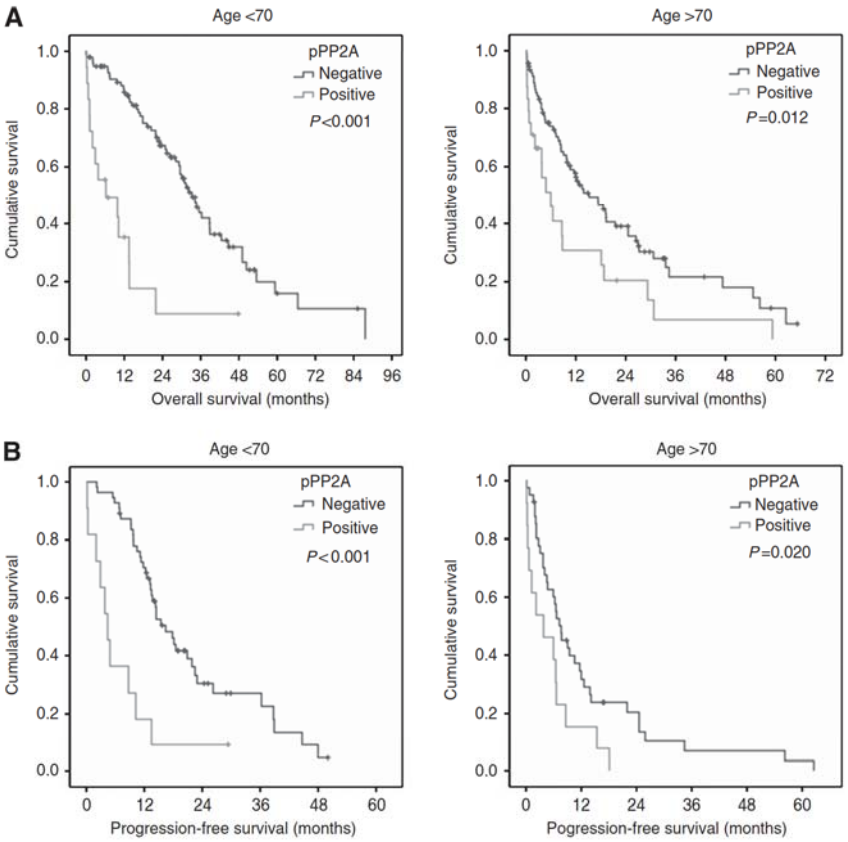


Figure 2. Kaplan–Meier analyses in the subgroups of patients aged < and >70 years: (**A**) Overall survival; (**B**) progression-free survival.

Table 2. Univariate and multivariate Cox analyses in the cohort of 243 patients with mCRC

	Univariate OS analysis			Multivariate OS Cox analysis		
	HR	95% CI Lower–upper	Significance	HR	95% CI Lower–upper	Significance
Age			**<0.001**			0.249
	1.000			1.000		
	1.875	1.352–2.599		1.250	0.855–1.827	
Gender			0.311		—	
Male	1.000					
Female	0.848	0.616–1.167		—	—	
Synchronous			0.118		—	
No	1.000					
Yes	1.301	0.935–1.811		—	—	
ECOG			**<0.001**			**<0.001**
0–1	1.000			1.000		
2–3	1.925	1.588–2.333		1.777	1.427–2.213	
MSI			0.450		—	
No	1.000					
Yes	1.281	0.674–2.437		—	—	
Number of metastatic sites			0.076		—	
1–2	1.000					
>2	1.250	0.977–1.600		—	—	
p-PP2A			**<0.001**			**<0.001**
No	1.000			1.000		
Yes	2.838	1.935–4.164		2.743	1.819–4.138	

Abbreviations: CI = confidence interval; HR = hazard ratio; OS = overall survival; MSI = magnetic source imaging; p-PP2A = protein phosphatase 2A. *P* values in bold font indicate differences statistically significant.

clinical relevance of p-PP2A in metastatic CRC we analysed its potential prognostic value by stratifying our cohort based on KRAS mutation status, and we found that p-PP2A retained its prognostic impact in both KRAS wild-type and KRAS mutated subgroups with a similar significance (Supplementary Figure 3). Importantly, multivariate analysis demonstrated that p-PP2A is an unfavourable independent factor associated with OS (hazard ratio 2.7; 95% confidence interval, 1.8–4.1; $P<0.001$) (Table 2) and PFS (hazard ratio 3.0; 95% confidence interval, 1.8–5.0; $P<0.001$) (Table 3) in metastatic CRC.

DISCUSSION

We report here that PP2A hyperphosphorylation is a recurrent molecular event in metastatic CRC associated with worse ECOG performance status and the presence of synchronous metastasis. Importantly, this alteration determines a markedly shorter overall and PFS, especially in the subgroup of patients younger than 70 years. The prognostic impact was similar in the KRAS wild-type and mutated subgroups. Moreover, multivariate analysis showed that high p-PP2A expression has an independent prognostic value for OS and PFS in patients with metastatic CRC. Of importance, our data provide strong evidence that p-PP2A has a potential prognostic value and could be a promising therapeutic target for future clinical trials using PP2A activators.

Despite progressive advances in our understanding of the molecular biology of CRC, patient outcomes in the metastatic subgroup are still very poor. Therefore, it is necessary to develop alternative therapeutic strategies to improve the survival of these patients. The tumour suppressor PP2A has been shown to be functionally inactivated in several types of human cancer through different contributing mechanisms, including the hyperphosphorylation of its catalytic subunit (Saydam *et al*, 2003; Cristobal *et al*, 2011). However, in comparison with other tumour models, the relevance of the tumour suppressor role of PP2A and its potential clinical significance in CRC remains mostly unknown. Therefore, to evaluate the clinical relevance of PP2A phosphorylation in metastatic CRC, we analysed the expression of p-PP2A in a cohort of 250 patients with metastatic CRC, observing high p-PP2A in 17.2% of cases (Table 1). The prevalence observed for this alteration in our cohort suggests that this would be a relevant molecular mechanism to inactivate PP2A in CRC.

Moreover, we observed that high p-PP2A correlated positively with a high grade of ECOG performance status and with the existence of synchronous metastasis at diagnosis in our cohort (Table 1). These results prompted us to hypothesise that this could be a molecular alteration characteristic of the advanced stages of CRC that could therefore have a prognostic value in patients with metastatic disease. In concordance with this, we observed that the subgroup of patients with high p-PP2A showed a substantially shorter OS and PFS compared with the low-p-PP2A subgroup (Figure 1), confirming the clinical relevance of p-PP2A in metastatic CRC. Moreover, the fact that the prognostic impact of p-PP2A showed higher significance in the subgroup of patients aged <70 years (Figure 2) is very interesting since this subgroup includes cases with more options from a therapeutic perspective that could benefit from the treatment with PP2A activating drugs

Table 3. Univariate and multivariate Cox analyses in the cohort of 243 patients with mCRC

	Univariate PFS analysis			Multivariate PFS Cox analysis		
	|HR	95% CI Lower–upper	Significance	HR	95% CI Lower–upper	Significance
Age			**0.001**			0.096
	1.000			1.000		
	1.966	1.318–2.932		1.476	0.933–2.333	
Gender			0.595		—	
Male	1.000					
Female	0.897	0.601–1.339		—	—	
Synchronous			0.056		—	
No	1.000					
Yes	1.522	0.990–2.341		—	—	
ECOG			**<0.001**			**0.012**
0–1	1.000			1.000		
2–3	1.556	1.224–1.978		1.420	1.081–1.866	
MSI			0.426		—	
No	1.000					
Yes	1.445	0.584–3.577		—	—	
Number of metastatic sites			**0.029**			**0.003**
1–2	1.000			1.000		
>2	1.374	1.033–1.827		1.564	1.165–2.099	
p-PP2A			**<0.001**			**<0.001**
No	1.000			1.000		
Yes	3.046	1.885–4.822		3.008	1.804–5.016	

Abbreviations: CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; MSI = magnetic source imaging; PFS = progression-free survival; p-PP2A = protein phosphatase 2A. *P* values in bold font indicate differences statistically significant.

(e.g. FTY720), which have shown promising effects in other cancers (Kalev and Sablina, 2011; Perrotti and Neviani, 2013).

As the mutation status of KRAS has a strong predictive value of response to cetuximab in CRC, we stratified our series by KRAS mutation status and observed that p-PP2A had a similar prognostic impact in both KRAS wild-type and KRAS mutated subgroups (Supplementary Figure 3). This result would indicate that the treatment with PP2A activators could also be a therapeutic option for those patients with high p-PP2A in whom cetuximab is not recommended or in those cases with cetuximab resistance. Interestingly, it has recently been reported that FTY720 could resensitise CRC cells to cetuximab, indicating a potential therapeutic relevance for FTY720 in metastatic CRC (Rosa *et al*, 2013). Our group has recently reported overexpression of the PP2A inhibitors SET and CIP2A and downregulation of PP2A regulatory subunits such as PPP2R2A and PPP2R5E as the contributing mechanisms to PP2A inhibition in CRC (Cristóbal *et al*, 2014); some of these alterations could be present in the low-p-PP2A subgroup of CRC patients. Therefore, additional investigations are needed to clarify the presence and potential clinical significance of these other PP2A-inactivating mechanisms in the subgroup of CRC patients with low p-PP2A levels. Furthermore, it has been reported that PP2A downregulation is a relevant event in some human cancers such as acute myeloid leukaemia and prostate cancer (Cristobal *et al*, 2011; Bhardwaj *et al*, 2014). However, our previous investigations did not show PP2A downregulated in CRC, suggesting that this alteration, if present, would be of low prevalence in CRC. Anyway, it would be of interest to further analyzed in future studies whether this alteration could be present in a subgroup of CRC patients.

In conclusion, we show that p-PP2A is a common alteration with clinical significance in metastatic CRC. Of importance, p-PP2A could serve as a novel molecular target that can help define a subgroup of metastatic CRC patients with worse outcome that could benefit by the future incorporation of PP2A-activating drugs in anticancer protocols.

ACKNOWLEDGEMENTS

This study was funded by the Biobank of Fundación Jiménez Díaz (FJD Biobank) (RD12/0036/0021), and PI12/01552 and PI13/02609 grants from Instituto de Salud Carlos III FEDER and S2010/BMD2344. R Manso is supported by the Fundación Conchita Rábago de Jiménez Díaz.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Arino J, Woon CW, Brautigan DL, Miller Jr TB, Johnson GL (1988) Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc Natl Acad Sci USA* **85**(12): 4252–4256.

Bhardwaj A, Singh S, Srivastava SK, Arora S, Hyde SJ, Andrews J, Grizzle WE, Singh AP (2014) Restoration of PPP2CA expression

reverses epithelial-to-mesenchymal transition and suppresses prostate tumour growth and metastasis in an orthotopic mouse model. *Br J Cancer* **110**(8): 2000–2010.

Chen J, Martin BL, Brautigan DL (1992) Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science* **257**(5074): 1261–1264.

Cristobal I, Blanco FJ, Garcia-Orti L, Marcotegui N, Vicente C, Rifon J, Novo FJ, Bandres E, Calasanz MJ, Bernabeu C, Odero MD (2010) SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* **115**(3): 615–625.

Cristobal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD (2011) PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. *Leukemia* **25**(4): 606–614.

Cristóbal I, Manso R, Rincón R, Caramés C, Senin C, Borrero A, Martínez-Useros J, Rodriguez M, Zazo S, Aguilera O, Madoz-Gúrpide J, Rojo F, García-Foncillas J (2014) PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. *Mol Cancer Ther* **13**(4): 938–947.

Eichhorn PJ, Creyghton MP, Bernards R (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* **1795**(1): 1–15.

Generali D, Buffa FM, Berruti A, Brizzi MP, Campo L, Bonardi S, Bersiga A, Allevi G, Milani M, Aguggini S, Papotti M, Dogliotti L, Bottini A, Harris AL, Fox SB (2009) Phosphorylated ERalpha, HIF-1alpha, and MAPK signaling as predictors of primary endocrine treatment response and 470 resistance in patients with breast cancer. *J Clin Oncol* **27**(2): 227–234.

Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signalling. *Cell* **80**(2): 225–236.

Janssens V, Goris J (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* **353**(Pt3): 417–439.

Janssens V, Goris J, Van Hoof C (2005) PP2A: the expected tumor suppressor. *Curr Opin Genet Dev* **15**(1): 34–41.

Kalev P, Sablina AA (2011) Protein phosphatase 2A as a potential target for anticancer therapy. *Anticancer Agents Med Chem* **11**(1): 38–46.

Kumar A, Pandurangan AK, Lu F, Fyrst H, Zhang M, Byun HS, Bittman R, Saba JD (2012) Chemopreventive sphingadienes downregulate Wnt signaling via a PP2A/Akt/GSK3β pathway in colon cancer. *Carcinogenesis* **33**(9): 1726–1735.

Lin SP, Lee YT, Yang SH, Miller SA, Chiou SH, Hung MC, Hung SC (2012) Colon cancer stem cells resist antiangiogenesis therapy-induced apoptosis. *Cancer Lett* **328**(2): 226–234.

McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics (2005) Reporting recommendations for tumor marker prognostic studies. *J Clin Oncol* **23**(36): 9067–9072.

Millward TA, Zolnierowicz S, Hemmings BA (1999) Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* **24**(5): 186–191.

Mumby M (2007) PP2A: unveiling a reluctant tumor suppressor. *Cell* **130**(1): 21–24.

Perrotti D, Neviani P (2008) Protein phosphatase 2A (PP2A), a drugable tumor suppressor in Ph1(+) leukemias. *Cancer Metastasis Rev* **27**(2): 159–168.

Perrotti D, Neviani P (2013) Protein phosphatise 2A: a target for anticancer therapy. *Lancet Oncol* **14**(6): e229–e238.

Rosa R, Marciano R, Malapelle U, Formisano L, Nappi L, D'Amato C, D'Amato V, Damiano V, Marfè G, Del Vecchio S, Zannetti A, Greco A, De Stefano A, Carlomagno C, Veneziani BM, Troncone G, De Placido S, Bianco R (2013) Sphingosine kinase 1 overexpression contributes to cetuximab resistance in human colorectal cancer models. *Clin Cancer Res* **19**(1): 138–147.

Ruediger R, Pham HT, Walter G (2001) Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. *Oncogene* **20**(15): 1892–1899.

Saydam G, Aydin HH, Sahin F, Selvi N, Oktem G, Terzioglu E, Buyukkececi F, Omay SB (2003) Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells. *Leuk Res* **27**(8): 709–717.

Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S (2000) Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. *Gut* **47**(2): 268–271.

Tamaki M, Goi T, Hirono Y, Katayama K, Yamaguchi A (2004) PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. *Oncol Rep* **11**(3): 655–659.

Tan J, Lee PL, Li Z, Jiang X, Lim YC, Hooi SC, Yu Q (2010) B55β-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. *Cancer Cell* **18**(5): 459–471.

Voronkov M, Braithwaite SP, Stock JB (2011) Phosphoprotein phosphatase 2A: a novel druggable target for Alzheimer's disease. *Future Med Chem* **3**(7): 821–833.

Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J, Evans GA (1998) Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* **282**(5387): 284–287.

Westermarck J, Hahn WC (2008) Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* **14**(4): 152–160.

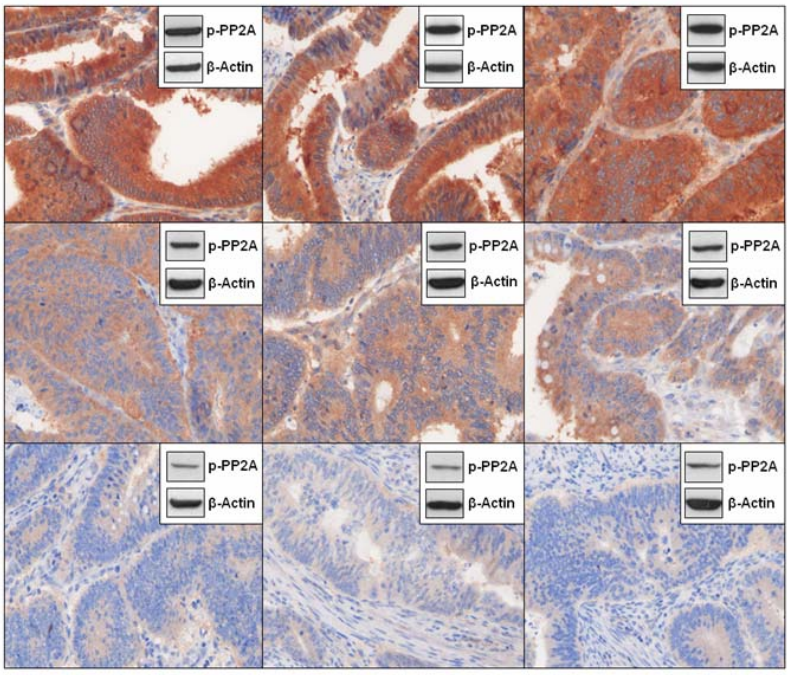
Xiong Y, Jing XP, Zhou XW, Wang XL, Yang Y, Sun XY, Qiu M, Cao FY, Lu YM, Liu R, Wang JZ (2013) Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation. *Neurobiol Aging* **34**(3): 745–756.

Zhou J, Pham HT, Ruediger R, Walter G (2003) Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J* **369**(Pt2): 387–398.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

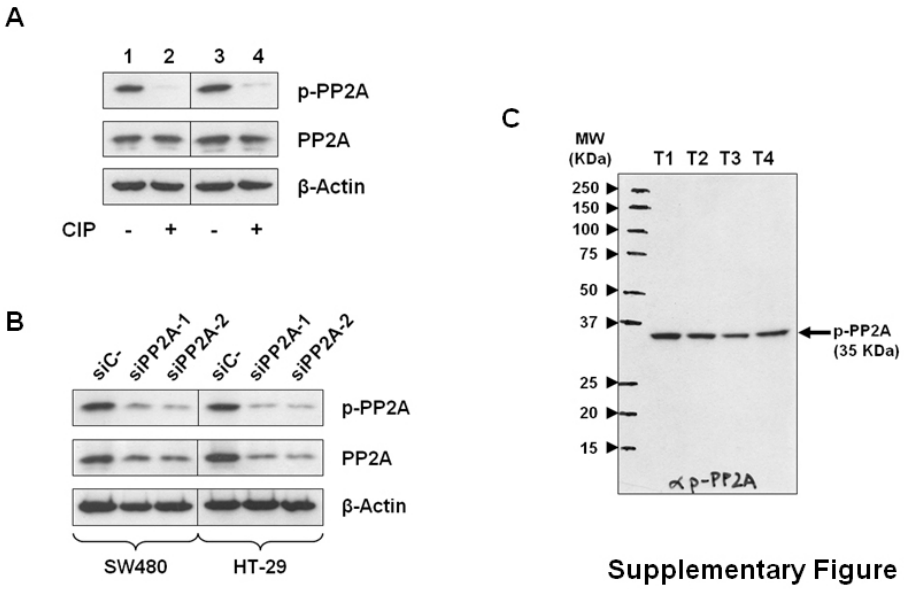
Titles and Legends to Supplementary Figures

Supplementary Figure 1. Immunohistochemical detection of p-PP2A showing different intensities of staining (magnification x400) together with western blot analyses.



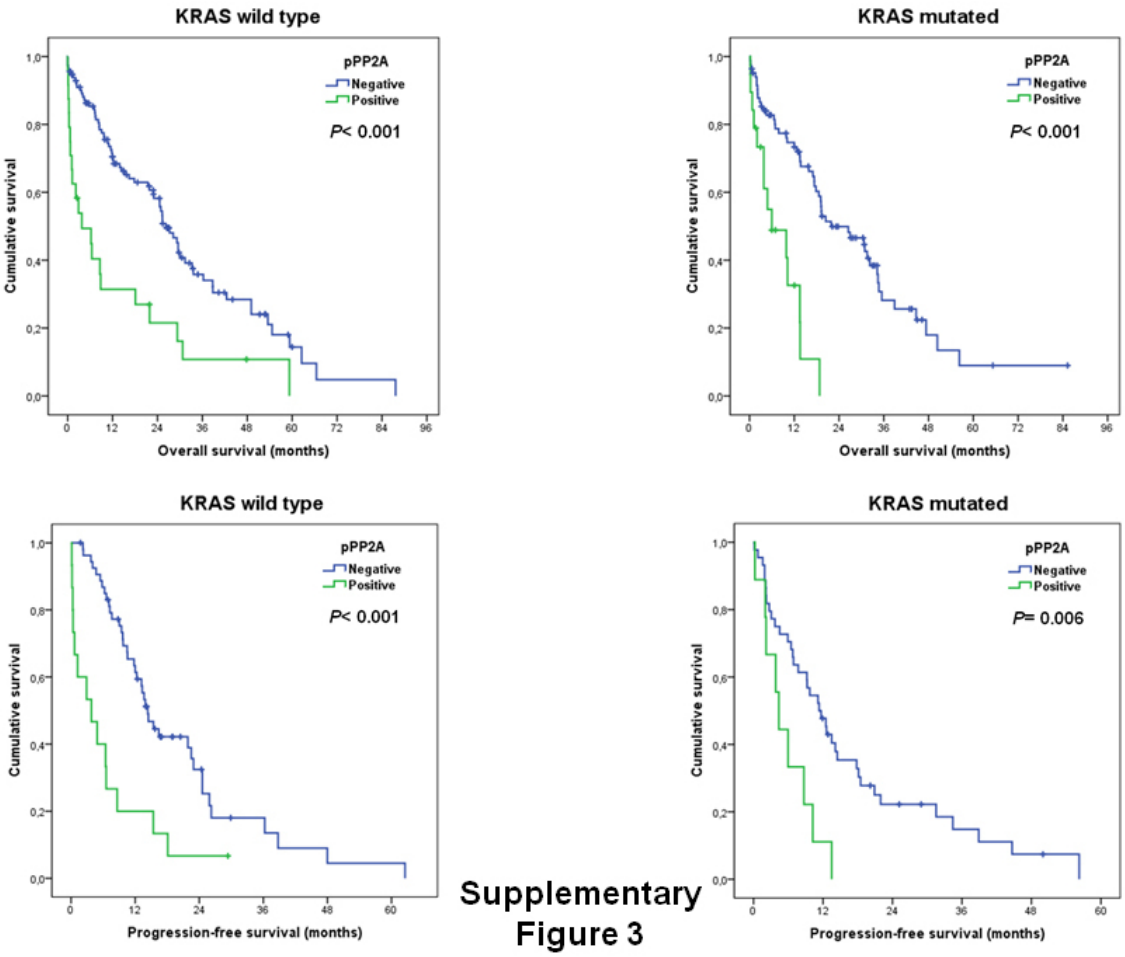
Supplementary Figure 1

Supplementary Figure 2. Experimental confirmation of the p-PP2A antibody specificity. (A) Western blot analysis showing loss of p-PP2A signal after dephosphorylation of protein extracts from SW480 (lines 1 and 2) and HT-29 (lines 3 and 4) cells using calf intestinal phosphatase; (B) PP2A expression levels and phosphorylation status in SW480 and HT-29 cells transfected with two different specific siRNAs against PP2A (siPP2A-1 and siPP2A-2) or a siRNA negative control (siC-); (C) Western blot analysis showing p-PP2A signal in four CRC tumor tissues (T1-4) as the unique major band recognized in the entire membrane.



Supplementary Figure 2

Supplementary Figure 3. Kaplan-Meier analyses of overall and progression-free survival in the subgroups of patients with KRAS wild type (n= 137) and KRAS mutated (n=102).



Supplementary Figure 3

Supplementary Table 1. Clinical and molecular characteristics of a series of 250 patients with metastatic CRC.

		No. (%)
Sex		
	Male	156 (62.4)
	Female	94 (37.6)
Age		
	<70	116 (50)
	≥70	116 (50)
	No data	18
ECOG		
	0-2	182 (81.2)
	3-4	42 (18.8)
	No data	26
MSI		
	No	226 (94.2)
	Yes	14 (5.8)
	No data	10
KRAS mutations		
	No	140 (56.9)
	Yes	106 (43.1)
	No data	4

Site of primary tumor		
	Cecum	24 (9.6)
	Right colon	37 (14.8)
	Transverse colon	10 (4)
	Left colon	21 (8.4)
	Sigma	73 (29.2)
	Rectum	85 (34)
Synchronous metastasis		
	No	85 (34.8)
	Yes	159 (65.2)
	No data	6
Number of metastatic sites		
	1-2	226 (90.4)
	>2	24 (9.6)
Liver metastasis		
	No	79 (32.4)
	Yes	165 (67.6)
	No data	6
Lung metastasis		
	No	166 (66.4)
	Yes	78 (33.6)
	No data	6
Lymph metastasis*		
	No	177 (72.5)
	Yes	67 (27.5)
	No data	6
Peritoneal metastasis		
	No	196 (80.3)
	Yes	48 (19.7)
	No data	6
Prior adjuvant chemotherapy**		
	No	33 (37.9)
	Yes	54 (62.1)
	No data	10
Treatment 1st line metastatic		
	Oxaliplatin	103 (43.3)
	Irinotecan	38 (16)
	5-FU	22 (9.2)
	Other	2 (0.8)
	None	73 (30.7)
	No data	12

*Non-regional lymph node involvement; **Cases with metachronous metastasis only

Article 3: Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation.[3]

Biochimica et Biophysica Acta 1842 (2014) 1823–1829

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation

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ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 25 June 2014
Accepted 26 June 2014
Available online 2 July 2014

Keywords:
PP2A-C phosphorylation
Colorectal cancer
Forskolin
5-Fluorouracil
Oxaliplatin

ABSTRACT

Background: The tumor suppressor protein phosphatase 2A (PP2A) is frequently inactivated in human cancer and phosphorylation of its catalytic subunit (p-PP2A-C) at tyrosine-307 (Y307) has been described to inhibit this phosphatase. However, its molecular and clinical relevance in colorectal cancer (CRC) remains unclear.
Methods: p-PP2A-C Y307 was determined by immunoblotting in 7 CRC cell lines and 35 CRC patients. CRC cells were treated with the PP2A activator forskolin alone or combined with the PP2A inhibitor okadaic acid, 5-fluorouracil and oxaliplatin. We examined cell growth, colonosphere formation, caspase activity and AKT and ERK activation.
Results: PP2A-C was found hyperphosphorylated in CRC cell lines. Forskolin dephosphorylated and activated PP2A, impairing proliferation and colonosphere formation, and inducing activation of caspase 3/7 and changes in AKT and ERK phosphorylation. Moreover, forskolin showed additive effects with 5-fluorouracil and oxaliplatin treatments. Analysis of p-PP2A-C Y307 in primary tumors confirmed the presence of this alteration in a subgroup of CRC patients.
Conclusions: Our data show that PP2A-C hyperphosphorylation is a frequent event that contributes to PP2A inhibition in CRC. Antitumoral effects of forskolin-mediated PP2A activation suggest that the analysis of p-PP2A-C Y307 status could be used to identify a subgroup of patients who would benefit from treatments based on PP2A activators.

1. Introduction

Colorectal cancer (CRC) is the third most common human cancer and a major cause of morbidity and mortality throughout the world [1]. CRC survival rates are similar between males and females, and the 5-year survival rate considering patients diagnosed from 2003 to 2009 is currently around 70%. Moreover, only 40% of CRC patients are diagnosed at a local stage of the disease, showing a 5-year survival rate of 90%. However, survival decreases to 70.4% and 12.5% in those CRC cases with regional and distant metastasis at diagnosis, respectively [2]. Despite progressive advances in the treatment of metastatic CRC through the development of novel cytotoxic drugs, patient outcomes are still very poor [3]. Therefore, it is necessary to better understand the molecular basis of this disease in order to develop alternative therapeutic strategies that may improve patient outcomes [4].

The loss of a correct equilibrium between kinase and phosphatase activities is a key event in cell transformation that deregulates normal cell homeostasis [5]. In fact, an aberrant kinase activity has been reported in many tumor types and different monotherapies based on kinase inhibitors have been incorporated into anticancer protocols. However, the potential therapeutic value of phosphatase activating drugs remains in comparison mostly unknown.

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that plays a key role as a tumor suppressor regulating many signaling pathways with a high relevance in human cancer through modulating the phosphorylation status of proteins such as AKT or ERK [6–9]. PP2A has been reported to be frequently inactivated in cancer cells by different molecular mechanisms including the phosphorylation

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http://dx.doi.org/10.1016/j.bbadis.2014.06.032
0925-4439/

of its catalytic subunit [PP2A-C] at Y307 [8–12]. In addition, its pharmacological activation has shown promising therapeutic effects, especially in leukemias [13–15]. Although several observations suggest that PP2A represents a drugable tumor suppressor also in CRC, it remains necessary to fully clarify the relevance of the different PP2A inhibitory mechanisms and the antitumor efficacy of PP2A activating drugs in this disease. In fact, low prevalent mutations affecting PP2A have been reported in CRC and our group has recently reported that PP2A inhibition is a common event in CRC and that its restoration using FTY720 (a FDA-approved immunosuppressor used in multiple sclerosis treatment with PP2A activating properties) shows promising antitumor effects [13,16–20]. Moreover, this phosphatase is involved in resistance of CRC cells to antiangiogenesis therapies [21,22]. Therefore, we hypothesized that PP2A would be commonly inhibited in CRC and that its phosphorylation on PP2A-C Y307 could represent a novel inhibitory mechanism with molecular and therapeutic relevance in this disease.

In this report, we show that PP2A-C hyperhosphorylation contributes to inactivate PP2A in CRC cells. Interestingly, we observed that forskolin treatment dephosphorylated and increased PP2A activity, impairing proliferation and colonosphere formation, and inducing activation of caspase 3/7 and changes in the phosphorylation status of AKT and ERK. Interestingly, forskolin showed additive effects with either 5-fluorouracil or oxaliplatin treatments. Moreover, we confirmed that PP2A-C is hyperphosphorylated in a subgroup of CRC patient samples, which indicates the potential therapeutic use of PP2A-C dephosphorylating drugs in those patients, alone or in combination with standard chemotherapy agents.

2. Materials and methods

2.1. Cell cultures

The human CRC cell lines RKO (ATCC CRL-2577), LoVo (ATCC CCL-229), SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38) and SW620 (ATCC CCL-227) were purchased from American Type Culture Collection (ATCC). Authentication was done by the authors (LGC Standards). Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS) and were grown at 37 °C in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). Cells were treated with the following reagents: 5-FU (1 μM) (Sigma), oxaliplatin (LOHP) (1 μM) (Sigma), forskolin (40 μM) (Calbiochem) and okadaic acid (OA) (2.5 nM) (Calbiochem). Forskolin has previously been reported to activate PP2A in association with its dephosphorylation [12], and OA has been used in this work at a concentration that inhibits PP2A but no other phosphatases [23].

2.2. Patient samples

The study comprised fresh frozen samples of 35 patients with CRC. Primary colorectal tissues were surgical resection specimens from CRC tumors obtained from Fundacion Jimenez Diaz Biobank (BFJD, Madrid). TNM (Tumor, Node, Metastases) staging was classified using the 7th American Joint Committee on Cancer (AJCC) staging system for CRC. Clinical data were collected from medical clinical records by an oncologist. Paired normal mucosa obtained from each patient was used as control. A pathologist confirmed that primary tumor tissues used in this work contained greater than 70% tumoral component. Informed consent was obtained from all patients. The ethical committee and institutional review board from the University Hospital "Fundacion Jimenez Diaz" approved the project.

2.3. Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following manufacturer's indications, clarified (12,000 ×g, 15 min, 4 °C), denatured and subjected to SDS-PAGE and Western-blot. Antibodies used were mouse monoclonal anti-PP2A (Upstate Inc.), rabbit monoclonal anti-p-PP2A-C Y307 (Epitomics), rabbit polyclonal anti-AKT, rabbit polyclonal anti-ERK (Cell Signaling Technology Inc.), rabbit polyclonal anti-pAKT^{Thr308}, rabbit polyclonal anti-pERK1/2$^{Thr202/Tyr204}$ (Santa Cruz Biotechnology) and mouse monoclonal anti-βactin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phospatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems). Densitometric analyses were performed using the Scion Image software (Scion Corporation). p-PP2A-C/PP2A-C ratios of CRC cell lines were normalized to normal control 3. For CRC patient samples, ratios were normalized to paired normal colonic mucosa in each CRC case.

2.4. Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer's indications.

2.5. PP2A phosphatase activity assays

Protein extracts were isolated from cell lysates or fresh frozen CRC patient samples (5–10 tissue sections of 15 μm/each) using TRIzol Reagent (Invitrogen). PP2A assays were performed with protein extracts (CRC cell lines: 50 μg; CRC patient samples: 100 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) and following the manufacturer's instructions. Briefly, PP2A-C was immunoprecipitated using 4 μg of PP2A-C antibody and 25 μl Protein A agarose slurry, both supplied by the kit. After 2 h of incubation in constant rocking, samples were washed 3 times with TBS 1X followed by one additional wash with a ser/thr assay buffer also provided by the kit. Next, 60 μl of a diluted phosphopeptide at 750 μM and 20 μl of ser/thr assay buffer were added, and the mix was incubated for 10 min at 30 °C in a shaking incubator, and then 25 μl of the mix was transferred into each well of a 96-well plate. Each measurement was performed in triplicates. 100 μl of Malachite Green Detection Solution was added, and the mix was incubated for 15 min at room temperature. Absorbance at 650 nm was used to calculate the amount of phosphate released (pmol) using a standard curve (0–2000 pmol). The PP2A activity measurements in tumor samples of the CRC patients included in this study (Table 1) are relative to paired normal colonic mucosa for each CRC patient.

2.6. Analysis of caspase activation

Quantification of caspase 3/7 activities in untreated or forskolin-treated CRC cells were carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5×10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase 3/7 substrate containing a tetrapeptide Asp-Glu-Val-Asp, was added with a 1:1 ratio of reagent to sample. After 90 min at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity in forskolin-treated cells compared with untreated cells are expressed as fold-change in luminescence.

2.7. Colonospheres

For the generation of colonospheres, 10,000 cells were plated in 6-well ultra-low attachment plates (Corning). CRC cell were grown in serum-free medium DMEM/F12 + GlutMAX™-I (Gibco) containing 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/ml human FGF (Sigma) and 50 ng/ml EGF (Sigma). After 7 days, plates were analyzed for colonosphere formation. For quantification of the number of cells per colonosphere, colonospheres were collected and dissociated with

Table 1
Clinical and molecular characteristics of the 35 CRC patients included in the study.

Case	Sex	Age	Site	Grade	T	N	Basal CEA	Neoadjuvant chemotherapy	High PP2A-C	PP2A activity
P1[a]	M	62	Rectum	G2	T1	N0	2.7	No	No	Inhibited
P2[a]	M	83	Sigma	G2	T3	N2	ND	No	Yes	Inhibited
P3[a]	M	73	Right colon	G2	T3	N0	3.1	No	Yes	Inhibited
P4[a]	M	85	Rectum	G2	T3	N1	4.2	No	No	Normal
P5[a]	M	77	Sigma	G2	T3	N0	ND	No	Yes	Inhibited
P6[a]	M	77	Right colon	G2	T3	N2	ND	No	No	Inhibited
P7[a]	M	77	Left colon	G2	T2	N0	1.3	No	No	Inhibited
P8[a]	M	85	Left colon	G2	T3	N0	2.8	No	No	Inhibited
P9[a]	M	71	Rectum	G2	T2	N0	ND	No	Yes	Inhibited
P10[a]	M	65	Sigma	G2	T3	N0	2.2	No	No	Inhibited
P11[a]	F	77	Rectum	G2	T2	N0	3	No	No	Inhibited
P12[a]	F	51	Rectum	G2	T2	N0	ND	No	No	Inhibited
P13[a]	F	57	Left colon	G2	T2	N0	0.5	No	No	Inhibited
P14[a]	F	70	Rectum	G1	T2	N1	129	Yes	No	Inhibited
P15[a]	M	80	Right colon	G2	T3	N0	4.4	No	No	Inhibited
P16[a]	F	51	Rectum	G2	T4	N2	ND	No	No	Inhibited
P17[a]	M	87	Right colon	G2	T3	N0	ND	No	No	Inhibited
P18[a]	M	66	Right colon	G1	T2	N0	3.2	No	No	Inhibited
P19[a]	M	52	Transverse colon	G2	T2	N0	3.9	No	Yes	Inhibited
P20[a]	M	64	Sigma	G2	T3	N0	1	No	No	Normal
P21[a]	F	85	Right colon	G2	T3	N0	ND	No	No	Inhibited
P22	M	81	Rectum	G1	T3	N2	60.4	No	No	Normal
P23	M	65	Transverse colon	G2	T2	N0	ND	No	Yes	Inhibited
P24	M	77	Rectum	G2	T3	N0	ND	No	No	Inhibited
P25	M	72	Transverse colon	G1	T2	N0	13.2	No	No	Inhibited
P26	M	91	Left colon	G2	T2	N0	1.9	No	Yes	Inhibited
P27	F	78	Right colon	G1	T2	N0	14.3	No	No	Normal
P28	M	75	Sigma	G2	T3	N0	ND	No	No	Inhibited
P29	M	78	Left colon	G2	T2	N0	1.2	No	No	Inhibited
P30	F	59	Left colon	G2	T3	N0	ND	No	Yes	Inhibited
P31	M	90	Right colon	G1	T3	N0	ND	No	Yes	Inhibited
P32	M	64	Left colon	G2	T3	N0	1.9	No	No	Inhibited
P33	F	87	Left colon	G1	T4	N1	ND	No	Yes	Inhibited
P34	F	73	Right colon	G2	T3	N0	1.2	No	No	Normal
P35	F	76	Right colon	G1	T3	N0	ND	No	Yes	Inhibited

M: male; F: female; ND: no data.
[a] These cases were previously included in Cristobal et al. (2014) [20].

trypsin to give single cell suspensions. Viable cells were counted in a Neubauer chamber using a trypan blue exclusion test.

2.8. Statistical analysis

Data represented are mean of three independent experiments ± s.d. Statistical comparisons were carried out by 2-sided *t*-test analyses. A *P* value less than 0.05 was considered statistically significant. Chou-Talalay analysis was performed after 72 h of treatment using the CompuSyn Software (ComboSyn, Inc.) and combination index (CI) was used to determine additive/synergism calculations between forskolin and 5-fluorouracil/oxaliplatin treatments. CI < 0.90, 0.90 < CI < 1.10 and CI > 1.10 were considered, respectively, synergistic, additive and antagonistic effects.

3. Results

3.1. PP2A-C is hyperphosphorylated in CRC cell lines

Inhibition of the tumor suppressor PP2A has been described in several human cancers and phosphorylation of PP2A-C at Y307 has been reported to be responsible for PP2A inactivation [10]. Therefore, we first analyzed the p-PP2A-C Y307 status in 7 CRC cell lines and in 3 normal colonic mucosa samples, observing an increased p-PP2A-C/PP2A-C ratio in all the 7 CRC cell lines analyzed in comparison with normal controls (Fig. 1). These results suggest that CRC cell lines present a reduced PP2A activity and that its hyperphosphorylation could be a relevant mechanism to inhibit PP2A in CRC.

3.2. Forskolin leads to a reduced proliferation dependent on PP2A activation

To study the biological relevance of p-PP2A-C Y307 we assessed proliferation of CRC cells after the treatment with the PP2A activator forskolin in the SW480 and HT-29 cell lines. We first confirmed by PP2A assays that forskolin activates PP2A, observing around 1.5-fold increase in the PP2A activity (Fig. 2A). As a control, we pretreated SW480 and HT-29 cells with the PP2A inhibitor OA for 2 h, followed by incubation with vehicle (DMSO) or forskolin for 48 h, observing that forskolin-induced PP2A activity was inhibited by OA (Fig. 2A). OA was not removed from the culture medium before the addition of forskolin. Similar results were obtained using DLD-1 cells (data not shown).

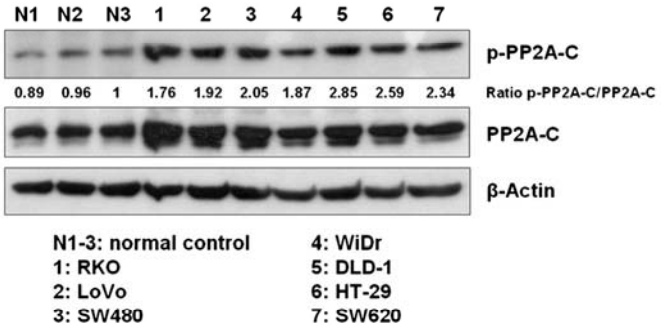


Fig. 1. Analysis of PP2A-C in CRC cell lines. Western blot analysis of PP2A-C phosphorylation and expression in normal mucosa and in 7 CRC cell lines, including a densitometric analysis of the p-PP2A-C/PP2A-C ratios, which were normalized to normal control 3 (N3). N1-3: normal mucosa.

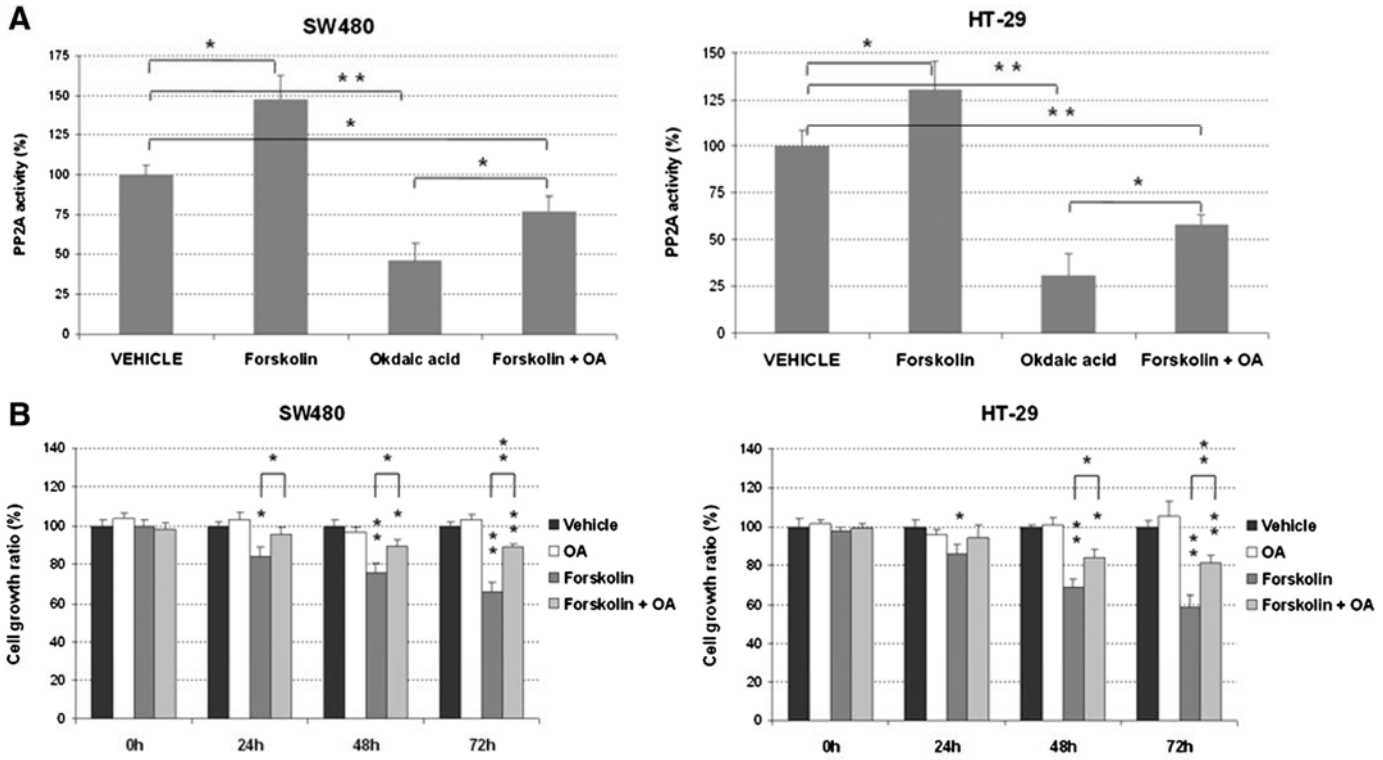


Fig. 2. Forskolin impairs cell proliferation via PP2A activation. (A) PP2A assays in SW480 and HT-29 cells treated with forskolin (40 μM; 48 h) and pretreated or not with OA for 2 h (2.5 nM). (B) MTS assay showing cell viability in SW480 and HT-29 cells treated with forskolin (40 μM) alone or in combination with OA (2.5 nM); $^{*}P < 0.05$; $^{**}P < 0.01$.

We next analyzed the effect of forskolin on cell growth, observing a decreased proliferation in forskolin-treated SW480 and HT-29 cells compared with vehicle-treated cells (Fig. 2B). These results were confirmed using DLD-1 cells (data not shown). In addition, we observed that the antiproliferative effects of forskolin were partially rescued by the pre-treatment with OA. Altogether, these results show that PP2A activation by forskolin decreases proliferation in CRC cells.

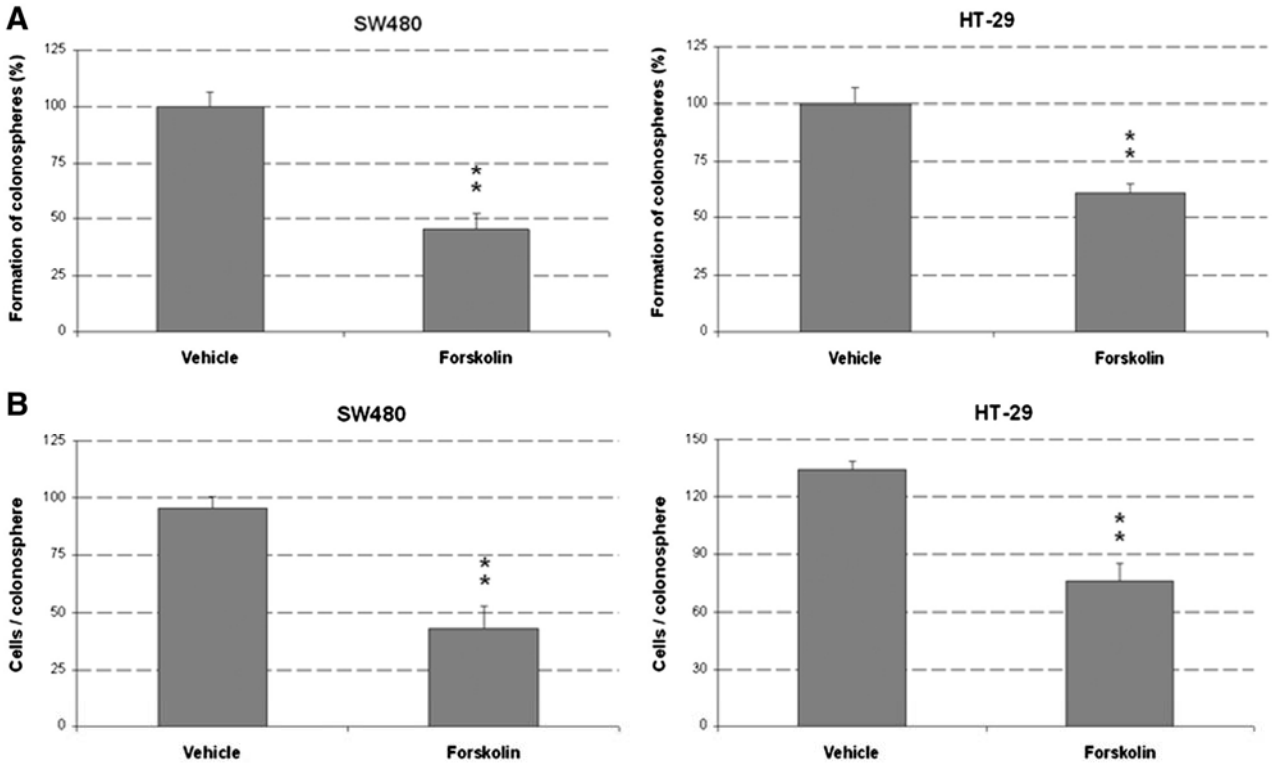


Fig. 3. Forskolin-induced PP2A activation reduces colonosphere formation capability in CRC cells. (A) Colonosphere formation (7 days) and (B) evaluation of the number of cells per colonosphere after forskolin treatment (40 μM) in SW480 and HT-29 cells; $^{*}P < 0.05$; $^{**}P < 0.01$.

3.3. Forskolin impairs colonosphere formation and promotes apoptosis in CRC cells

To further investigate the molecular effects of the forskolin-induced PP2A activation in CRC, we assessed colonosphere formation in SW480 and HT-29 cells treated with forskolin. Interestingly, we observed that forskolin led to decreased colonosphere formation ability in both the number and size of colonospheres formed in both cell lines (Fig. 3 and Supplementary Fig. 1). These observations were confirmed in DLD-1 cells (data not shown).

Furthermore, we assessed apoptosis in SW480 and HT-29 cells after forskolin treatment. Vehicle-treated cells were used as controls. Consistent with its ability to enhance PP2A activity and impair cell growth (Fig. 2), forskolin induced an activation of caspase 3/7 in SW480 cells (Fig. 4A). Results were confirmed in the HT-29 (Fig. 4A), and DLD-1 (data not shown) cell lines. Moreover, caspase activity decreased when cells were pretreated with OA.

3.4. Forskolin induces changes in the phosphorylation status of PP2A targets

We next analyzed by western blot the molecular effects of the forskolin-induced PP2A activation, observing that p-PP2A-C Y307 was negatively affected in forskolin-treated SW480 and HT-29 cells in comparison with cells treated with vehicle (DMSO). Interestingly, forskolin also decreased phosphorylation (activity) of AKT and ERK without affecting their expression levels (Fig. 4B). Pretreatment with OA restored forskolin-induced dephosphorylation in all cases. Similar results were obtained with the DLD-1 cell line (data not shown). These results indicate that forskolin-induced PP2A-C dephosphorylation and activation increases apoptosis and affects phosphorylation status of PP2A targets in CRC cells.

3.5. Additive/synergistic effects of forskolin-induced PP2A activation with 5-FU and oxaliplatin treatments in CRC cells

In order to assess the effect of a combination between standard induction chemotherapy drugs used in CRC and forskolin, we treated CRC cells with either 5-FU or LOHP, alone or in combination with forskolin. Interestingly, we observed that forskolin enhanced the antitumor effects mediated by 5-FU (Fig. 5A) and LOHP (Fig. 5B) treatments in both SW480 and HT-29 cell lines. These data were also confirmed in DLD-1 (data not shown). Chou-Talalay analyses showed that the forskolin/5-FU combination has additive effects in SW480 cells (CI = 1.08). Moreover, synergistic antitumor effects were observed when forskolin was combined with either 5-FU in HT-29 cells (CI = 00.61) or LOHP in SW480 (CI = 0.88) and HT-29 cells (CI = 0.62). Altogether, these results indicate that forskolin treatment potentiates antitumor activity of either 5-FU or LOHP treatments in CRC cells.

3.6. Analysis of PP2A phosphorylation status in CRC patient samples

To further evaluate the importance of p-PP2A-C Y307 in CRC, we studied by western blot the phosphorylation status of PP2A in 35 cases with CRC. Patient characteristics are included in Table 1.

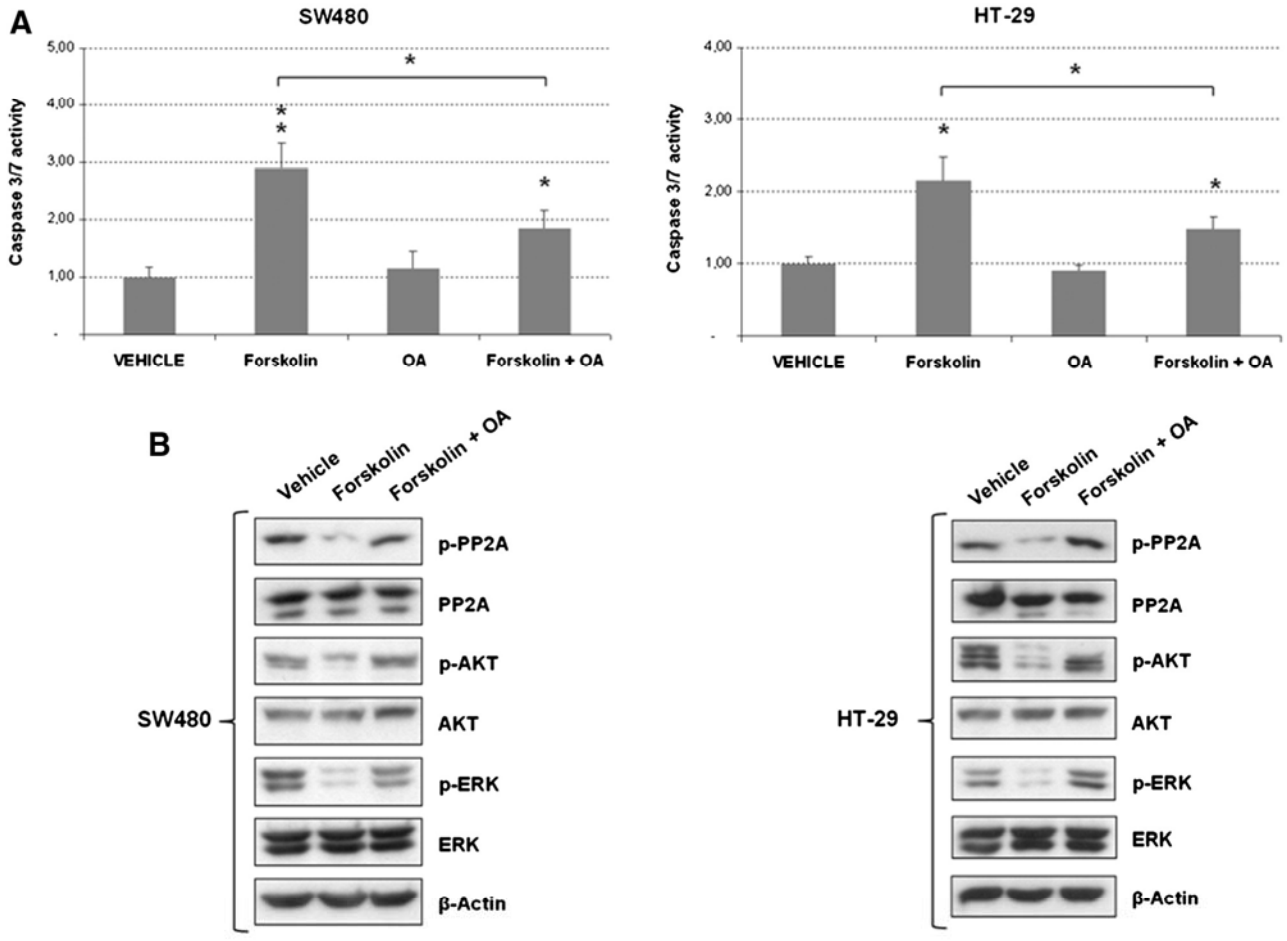


Fig. 4. Forskolin induces caspase-dependent apoptosis together with changes in the phosphorylation status of AKT and ERK. (A) Caspase 3/7 assays in SW480 and HT-29 cells untreated (DMSO), and forskolin-treated (40 μM; 48 h) alone or in combination with a pretreatment with OA for 2 h (2.5 nM). (B) Western blot showing PP2A-C, AKT and ERK1/2 phosphorylation and expression after forskolin treatment (40 μM; 48 h) in SW480 and HT-29 cells; $^{*}P < 0.05$; $^{**}P < 0.01$.

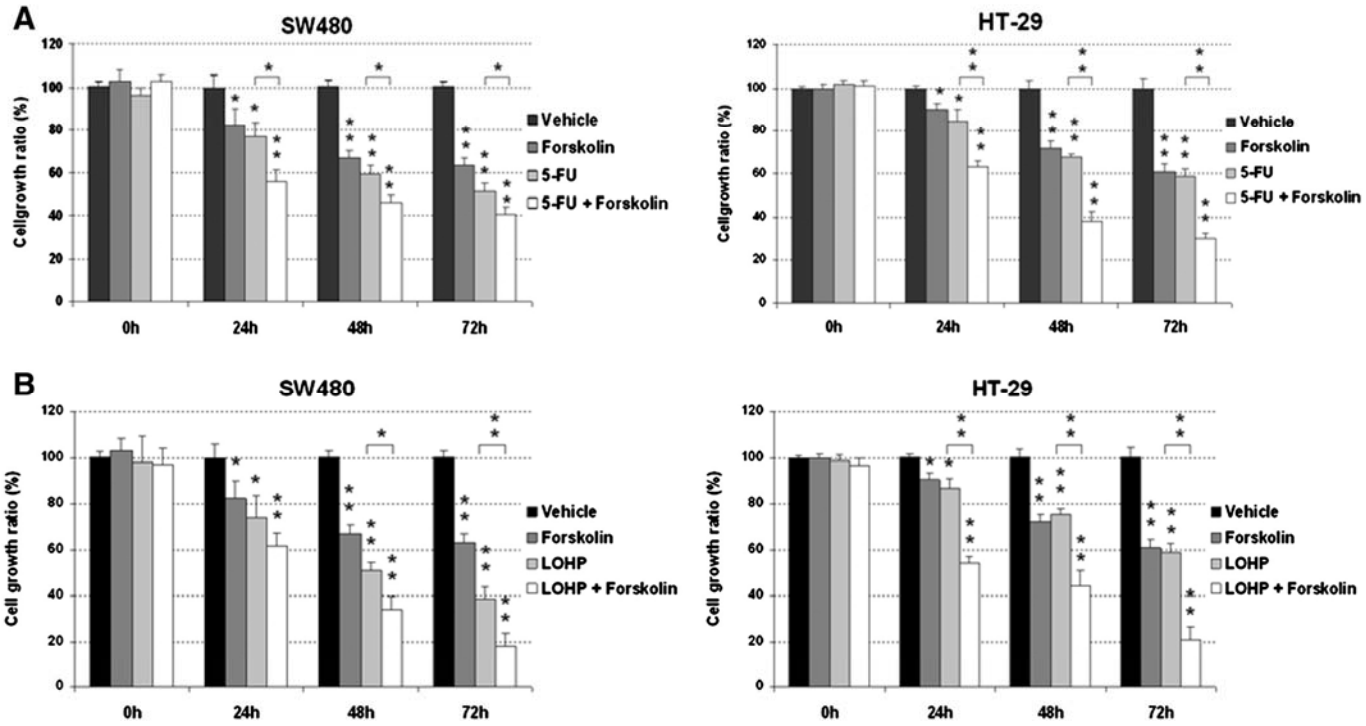


Fig. 5. Forskolin shows an additive/synergistic effects with 5-FU and LOHP treatments in CRC cells. MTS assays showing the effect of forskolin (40 μM) and 5-FU (1 μM) (A) or LOHP (1 μM) (B) alone or in combination in SW480 and HT-29 cells. Untreated cells (DMSO) were used as controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

Interestingly, we found that 11 CRC patients showed higher p-PP2A-C Y307 levels in tumor tissues compared with paired normal colonic mucosa (Fig. 6 and Supplementary Fig. 2). PP2A assays showed low PP2A activity in 30 out of 35 CRC patients. Of importance, PP2A was found inhibited in all the 11 cases with high p-PP2A-C levels (Table 1). These results would indicate that PP2A-C is hyperphosphorylated in a subgroup of CRC patients.

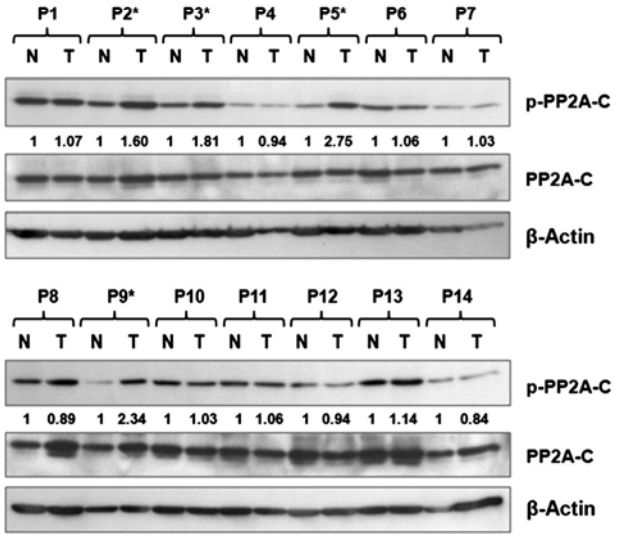


Fig. 6. Analysis of p-PP2A-C in CRC patients. Western blot analysis of PP2A-C phosphorylation and expression in CRC patient samples, including a densitometric analysis of the p-PP2A-C/PP2A-C ratio. Ratios were normalized to paired normal colonic mucosa in each CRC case; N: normal mucosa; T: tumor sample; *CRC patients with high p-PP2A-C levels.

4. Discussion

We report here that p-PP2A-C Y307 is a recurrent alteration that contributes to inactivate PP2A in CRC cells. The treatment with forskolin increased PP2A activity, affecting proliferation, colonosphere formation and activation of caspase 3/7 in CRC cells and leading to changes in the activation status of AKT and ERK. Interestingly, we observed that forskolin-induced PP2A activation showed promising therapeutic effects in combination with standard induction chemotherapy drugs such as 5-FU or LOHP. Moreover, we confirmed that PP2A-C hyperphosphorylation is an alteration present in a subgroup of CRC patients. Of importance, our data provides strong evidences that p-PP2A-C could be a promising therapeutic target in CRC.

PP2A is a well established tumor suppressor functionally inactivated in several types of human cancer through different contributing mechanisms including, in the case of CRC, mutations affecting PP2A subunits that impair the formation of the heterotrimeric PP2A complex then inhibiting its phosphatase activity [10,16–19]. However, these mutations have been reported to show a low prevalence in CRC, which suggests that other different alterations could be more relevant to inhibit PP2A in this disease. Another molecular inactivating mechanism is the phosphorylation of PP2A-C at Y307 [10]. In fact, PP2A-C hyperphosphorylation has been previously described as relevant in other malignancies such as acute myeloid leukemia [12] or Alzheimer's disease [24]. In order to evaluate the potential relevance of this mechanism in CRC we analyzed the p-PP2A-C status in 7 CRC cell lines, observing that all of them showed PP2A-C hyperphosphorylation, suggesting a biological significance of this alteration in the pathogenesis of CRC.

To further investigate the importance of p-PP2A-C in CRC we used forskolin to treat CRC cells. We considered the use of this drug because it has previously been reported to activate PP2A in association with its dephosphorylation [12]. Interestingly, forskolin-mediated PP2A activation induced a potent antitumoral effect in CRC cells, impairing cell growth and colonosphere formation, and increasing activation of caspase 3/7. Interestingly, we observed that forskolin-induced PP2A

activity and growth inhibition were partially restored by pretreatment with the PP2A inhibitor OA indicating that the antitumor effects induced by this drug are due preferentially to PP2A activation. However, the absence of a total restoration would indicate that PP2A activation is not the only molecular mechanism involved in the biological effects showed by this drug, and that probably cAMP is involved since its role as an inhibitor of cell proliferation has been described and forskolin is a cAMP activator [25]. As expected, we observed a PP2A-C dephosphorylation after treatment with forskolin without changes in PP2A expression, confirming that forskolin activates PP2A through p-PP2A-C Y307 dephosphorylation. Moreover, we observed that the molecular mechanism by which forskolin is acting involves the inhibition of the AKT and ERK, both PP2A targets, suggesting that this effect occurs via PP2A activation. In fact, a PP2A activation has been described which leads to similar effects on AKT and ERK activation status by the use of selenate and FTY720 in CRC. It has been reported that selenate reduces cell growth and induces apoptosis in DLD-1 cells. Of interest, selenate acts by activating PP2A, which inhibits the PI3K/AKT pathway. However, the use of selenate at very low concentrations unexpectedly promoted epithelial to mesenchymal transition and activated AKT, probably because PP2A activation failed at this low concentrations [26]. In addition, we recently described that FTY720-induced PP2A activation decreased both AKT and ERK phosphorylation status in CRC cells [26]. These observations are in concordance with the results obtained here with forskolin.

Interestingly, our results also showed that forskolin exhibits additive antitumor effects with the standard chemotherapy reagents 5-FU and LOHP, suggesting the use of PP2A activators such as forskolin for future trials in combination with these standard chemotherapy drugs. To confirm the importance of p-PP2A-C Y307 in CRC we analyzed 35 CRC patients (paired normal colonic mucosa and tumor samples), observing higher p-PP2A-C Y307 in 11 out of the 35 CRC patient samples, which suggest the existence of a subgroup of CRC patients with this alteration that would be good candidates to be treated with PP2A activators. Of relevance, 21 out of the 35 CRC patients included in this work were previously studied by our group in a recently published work about PP2A and its activating drug FTY720 in CRC [20]. Thus, we observe that other PP2A inhibitory alterations (e.g. overexpression of the endogenous PP2A inhibitors SET and CIP2A) can be present in CRC patients with low p-PP2A-C levels and lead to PP2A inhibition. On the other hand, the potential advantage of forskolin as opposed to other PP2A activating drugs (such as FTY720) is based on its molecular mechanism of action in CRC cells, since our group has recently reported that PP2A-C phosphorylation at Y307 is not affected by the treatment with FTY720 in CRC cells [20]. However, we describe here that high p-PP2A-C levels would serve to define a subgroup of CRC patients who are candidates to be treated with PP2A activating/dephosphorylating drugs such as forskolin.

In conclusion, we show that PP2A-C hyperphosphorylation represents a molecular mechanism to inactivate this tumor suppressor in CRC. Of importance, our results indicate the potential use of PP2A-C dephosphorylating/activating drugs alone or in combination with standard chemotherapy agents currently used in a subgroup of CRC patients. However, the relatively small number of CRC patients included in this work is a limitation of the study. Therefore, further studies are needed to fully clarify the relevance of p-PP2A-C Y0307 in CRC pathogenesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.06.032.

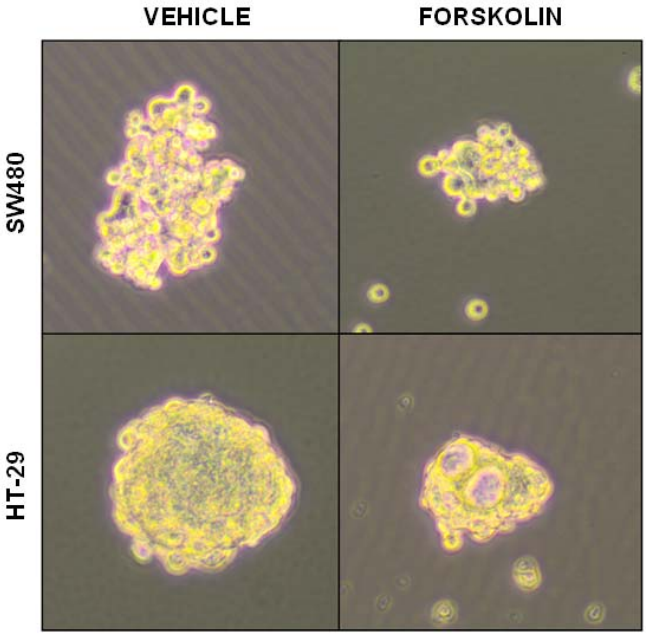
Funding

Biobank of Fundación Jiménez Díaz (FJD Biobank) (RD09/0076/00101) and PI12/01552 grants from "Instituto de Salud Carlos III FEDER" and S2010/BMD2344; R. Manso is supported by "Fundacion Conchita Rabago de Jiménez Diaz".

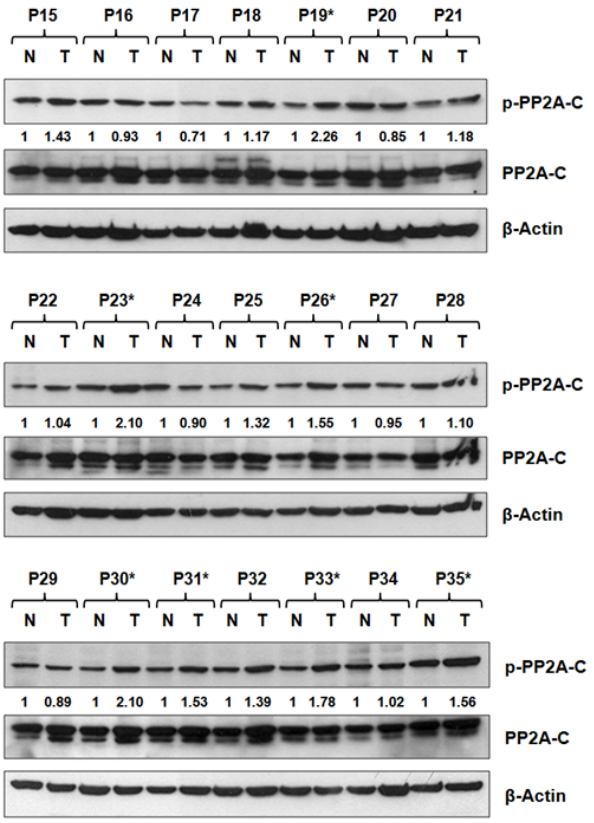
The authors declare no financial disclosures.

References

[1] A.S. Walker, N.P. Zwintscher, E.K. Johnson, et al., Future directions for monitoring treatment response in colorectal cancer, J. Cancer 5 (2014) 44–57.
[2] R. Siegel, C. Desantis, A. Jemal, Colorectal cancer statistics, CA Cancer J. Clin. 64 (2014) 104–117.
[3] M.H. Dietvorst, F.A. Eskens, Current and novel treatment options for metastatic colorectal cancer: emphasis on aflibercept, Biol. Ther. 3 (2013) 25–33.
[4] I. Ewing, J.J. Hurley, E. Josephides, A. Millar, The molecular genetics of colorectal cancer, Frontline Gastroenterol. 5 (2014) 26–30.
[5] T. Hunter, Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signalling, Cell 80 (1995) 225–236.
[6] P.J. Eichhorn, M.P. Creyghton, R. Bernards, Protein phosphatase 2A regulatory subunits and cancer, Biochim. Biophys. Acta 1795 (2009) 1–15.
[7] V. Janssens, J. Goris, C. Van Hoof, PP2A: the expected tumor suppressor, Curr. Opin. Genet. Dev. 15 (2005) 34–41.
[8] M. Mumby, PP2A: unveiling a reluctant tumor suppressor, Cell 130 (2007) 21–24.
[9] J. Westermarck, W.C. Hahn, Multiple pathways regulated by the tumor suppressor PP2A in transformation, Trends Mol. Med. 14 (2008) 152–160.
[10] J. Chen, B.L. Martin, D.L. Brautigan, Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation, Science 257 (1992) 1261–1264.
[11] I. Cristobal, F.J. Blanco, L. Garcia-Orti, et al., SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia, Blood 115 (2010) 615–625.
[12] I. Cristobal, L. Garcia-Orti, C. Cirauqui, M.M. Alonso, M.J. Calasanz, M.D. Odero, PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect, Leukemia 25 (2011) 606–614.
[13] D. Perrotti, P. Neviani, Protein phosphatase 2A (PP2A), a drugable tumor suppressor in Ph1(+) leukemias, Cancer Metastasis Rev. 27 (2008) 159–168.
[14] P. Kalev, A.A. Sablina, Protein phosphatase 2A as a potential target for anticancer therapy, Anticancer Agents Med. Chem. 11 (2011) 38–46.
[15] D. Perrotti, P. Neviani, Protein phosphatise 2A: a target for anticancer therapy, Lancet Oncol. 14 (2013) e229–e238.
[16] S.S. Wang, E.D. Esplin, J.L. Li, et al., Alterations of the PPP2R1B gene in human lung and colon cancer, Science 282 (1998) 284–287.
[17] Y. Takagi, M. Futamura, K. Yamaguchi, S. Aoki, T. Takahashi, S. Saji, Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers, Gut 47 (2000) 268–271.
[18] R. Ruediger, H.T. Pham, G. Walter, Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene, Oncogene 20 (2001) 1892–1899.
[19] M. Tamaki, T. Goi, Y. Hirono, K. Katayama, A. Yamaguchi, PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers, Oncol. Rep. 11 (2004) 655–659.
[20] I. Cristóbal, R. Manso, R. Rincón, et al., PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential, Mol. Cancer Ther. 13 (2014) 938–947.
[21] J. Tan, P.L. Lee, Z. Li, et al., B55β-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer, Cancer Cell 18 (2010) 459–471.
[22] S.P. Lin, Y.T. Lee, S.H. Yang, et al., Colon cancer stem cells resist antiangiogenesis therapy-induced apoptosis, Cancer Lett. 328 (2012) 226–234.
[23] G. Saydam, H.H. Aydin, F. Sahin, et al., Involvement of protein phosphatase 2A in interferon-alpha-2b-induced apoptosis in K562 human chronic myelogenous leukaemia cells, Leuk. Res. 27 (2003) 709–717.
[24] Y. Xiong, X.P. Jing, X.W. Zhou, et al., Zinc induces protein phosphatase 2A inactivation and tau hyperphosphorylation through Src dependent PP2A (tyrosine 307) phosphorylation, Neurobiol. Aging 34 (2013) 745–756.
[25] L. Gamet, J.C. Murat, A. Remaury, et al., Vasoactive intestinal peptide and forskolin regulate proliferation of the HT29 human colon adenocarcinoma cell line, J. Cell. Physiol. 150 (1992) 501–509.
[26] T. Tsukamoto, S. Hama, K. Kogure, H. Tsuchiya, Selenate induces epithelial-mesenchymal transition in a carcinoma colorectal carcinoma cell line by AKT activation, Exp. Cell Res. 319 (2013) 1913–1921.



Supplementary Figure 1



Supplementary Figure 2

Article 4: Deregulation of the PP2A inhibitor SET shows promising therapeutic implications and determines poor outcome in patients with metastatic colorectal cancer.[4]

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

Personalized Medicine and Imaging

Clinical Cancer Research

Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer

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Abstract

Purpose: SET is an endogenous PP2A inhibitor that might represent a novel molecular target for antitumor therapy. The aim of this study was to evaluate the molecular effects of SET deregulation and its potential clinical significance in metastatic colorectal cancer (mCRC).

Experimental Design: We studied the biologic effects of SET on cell growth, colonosphere formation, caspase activity, PP2A activation status, and sensitivity to oxaliplatin and FIY720 treatments. Moreover, we analyzed SET expression by immunostaining in 242 patients with mCRC.

Results: SET deregulation promotes cell growth and colonosphere formation and inhibits PP2A, thereby impairing its antitumor effects. Moreover, SET reduces sensitivity to oxaliplatin in colorectal cancer cell lines, which is restored after FIY720 treatment. SET overexpression was detected in 24.8% (60 of 242) of patients with mCRC and determined significantly shorter overall (8.6 vs. 27 months; $P < 0.001$) and progression-free survival (7.1 vs. 13.7 months; $P < 0.001$), and poor response to oxaliplatin-based chemotherapy ($P = 0.004$). Interestingly, its prognostic value was particularly evident in patients younger than 70 years and in those harboring KRAS mutations.

Conclusions: SET overexpression is a frequent event in mCRC that plays a potential oncogenic role associated with worse outcome and resistance to oxaliplatin. Moreover, this alteration defines a subgroup of patients who could benefit from therapies containing PP2A activators such as FIY720. *Clin Cancer Res; 21(2); 347–56. ©2014 AACR.*

Introduction

Colorectal cancer is the gastrointestinal cancer with the highest incidence, and the stage of the disease at the time of diagnosis is the most critical factor for patient outcomes (1). Moreover, progression to metastatic disease affects a large number of cases and it represents the subgroup of patients with the worst prognosis. Despite progressive clinical advances that have been carried out in the last decade to decrease or prevent metastasis, patient outcomes are still very poor (2). It is therefore a clinical challenge to develop alternative therapeutic strategies to improve the survival of these patients.

The protein SET is a PP2A inhibitor (3) that participates in the regulation of a wide variety of molecular processes (4–10). Of importance, SET plays an oncogenic role modulating signaling pathways with high relevance in human cancer (11). For instance, it has been reported that SET activates the transcription factor AP-1, deregulates AKT signaling, inhibits the DNase activity of the tumor-suppressor NM23-H1, or negatively regulates p53 acetylation, thus repressing its activity (12–16). Moreover, SET is overexpressed in several neoplasms (17–21) and it has been proposed as a novel molecular target for anticancer therapy (20–23). The transcription factor EVI-1 and the miR199b have been described to regulate SET expression in acute myeloid leukemia and choriocarcinoma (18, 24).

As indicated above, SET strongly inhibits PP2A, a tumor suppressor that regulates many signaling pathways, and whose loss of function is involved in cell transformation (11, 25–27). Different molecular strategies to inhibit PP2A have been described in transformed cells, including the overexpression of endogenous inhibitors such as SET. In fact, the inactivation of PP2A in human cancer seems to be a very recurrent and relevant event in human cancer and the potential therapeutic benefits of its pharmacologic activation has been investigated in the last years with very promising results (28–30). Interestingly, it has been reported that the antitumor effects showed by the PP2A activator FIY720 are mediated by SET in lung cancer (31). Moreover, our group has recently described that PP2A inhibition is a common event in colorectal cancer and that its restoration using FIY70 or forskolin induces promising antitumor effects (32, 33).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-14-0724

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

Translational Relevance

The SET oncogene represents a novel potential therapeutic target in human leukemia, but its potential relevance in solid tumors as colorectal cancer remains mostly unknown. Here, we show that SET deregulation is a recurrent alteration that contributes to inactivate PP2A and enhances tumor malignant properties of colorectal cancer cells. Interestingly, together with its prognostic value, SET is predictive of response to oxaliplatin-based chemotherapy backbone, which could have an important relevance in the current clinical practice. Thus, SET could differentiate a subgroup of patients who could benefit by the future incorporation of PP2A-activating drugs in anticancer protocols, alone or in combination with standard chemotherapy.

In this report, we further investigated the potential relevance of SET in colorectal cancer. Western blot analysis confirmed SET overexpression in colorectal cancer cell lines. Moreover, we observed that SET promotes cell growth, colonosphere formation, and restores the reduced cell viability induced after PP2A overexpression. Furthermore, we identified that SET induces a resistance in colorectal cancer cells to oxaliplatin that is impaired after FTY720 treatment. To determine its clinical relevance, we quantified SET in a series of 242 patients with metastatic colorectal cancer (mCRC), observing that SET overexpression is a recurrent molecular event that predicts shorter overall survival (OS), disease-free survival (DFS), and response to oxaliplatin-based chemotherapy.

Materials and Methods

Cell cultures and transfection

The human colorectal cancer cell lines SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38), SW620 (ATCC CCL-227), HCT-116 (ATCC CCL-247) and LS513 (ATCC CRL-2134) were purchased from the American Type Culture Collection (ATCC). Of note, the SW480 and SW620 cell lines are derived from the same patient. Authentication was done by the authors in all cases (LGC Standards). Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum and were grown at 37ºC in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/mL) and streptomycin (0.1 mg/mL). Cells were treated with oxaliplatin (LOHP; 1 μmol/L; Sigma) and FTY720 (10 μmol/L; Calbiochem) as previously reported (32–34). For transfection experiments, colorectal cancer cells were seeded in 6-well plates and transfected with 10 μL of Lipofectamine 2000 (Life Technologies) and 2 μg of plasmidic vectors or 75 nmol/L of SET-specific siRNAs designed and synthesized by Dharmacon RNA Technologies.

Patient samples

Primary colorectal tissues were surgical resection specimens from colorectal cancer tumors obtained from Fundacion Jimenez Diaz Biobank (BFJD, Madrid, Spain). The study comprised fresh-frozen samples of 14 patients with colorectal cancer with paired normal mucosa and tumor obtained from surgical specimens, consecutive FFPE tumor samples of 145 patients with colorectal cancer without mCRC, and 242 patients with metastatic disease who were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data, and metastatic disease. TNM (tumor–node–metastases) staging was classified using the 7th American Joint Committee on Cancer (AJCC) staging system for colorectal cancer. Clinical data were collected from medical clinical records by oncologists (J. García-Foncillas and C. Caramés). KRAS mutational status was determined by the Cobas KRAS Mutation Test Kit (Roche Molecular Diagnostics) following the manufacturer's procedures. Tissue microarrays (TMA) were constructed. Representative areas of each tumor were carefully selected and three tissue cores (1-mm diameter) were obtained using a TMA workstation (T1000 Chemicon). Samples were taken anonymously. The ethical committee and Institutional Review Board approved the project.

Western blot analysis

Protein extracts were isolated using TRizol Reagent (Invitrogen) following the manufacturer's indications, clarified (12,000 × *g*, 15 minutes, 4°C), denatured, and subjected to SDS-PAGE and Western blot analysis. Antibodies used were rabbit polyclonal anti-SET (Abcam) and mouse monoclonal anti-β-actin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phosphatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems).

Cell viability assay

Cell proliferation was measured in triplicate wells by the MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer's indications. IC_{50} was calculated using the SigmaPlot 11.0 bioinformatic tool.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described (28).

Analysis of caspase activation

Quantification of caspase-3/7 activities was carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5×10^3 cells were plated in a white-walled 96-well plate, and the Z-DEVD reagent, the luminogenic caspase-3/7 substrate containing a tetrapeptide Asp–Glu–Val–Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity are expressed as fold-change in luminescence.

Cell-cycle analysis

Cells were harvested, pelleted, and fixed in 70% ethanol on ice for 1 hour. After two washes in PBS, cell were treated with RNaseA for 30 minutes at 37ºC and stained with propidium iodide (25 μg/mL; BD Pharmigen) for 10 minutes at room temperature in dark conditions before the flow analysis.

Colonospheres

For the generation of colonospheres, 10,000 cells were plated in 6-well ultra-low attachment plates (Corning). Colorectal cancer

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

cell were grown in serum-free medium DMEM/F12+GlutMAX-I (Gibco) containing 1% N_2 (Gibco), 2% B27 (Gibco), 20 ng/mL human FGF (Sigma), and 50 ng/mL EGF (Sigma). After 7 days, plates were analyzed for colonosphere formation. For quantification of the number of cells per colonosphere, colonospheres were collected and dissociated with trypsin to give single-cell suspensions. Viable cells were counted in a Neubauer chamber using a Trypan Blue exclusion test.

Immunohistochemistry

Tissue sections (3 μm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 minutes. Slides were incubated with same primary antibody against SET as described for 60 minutes at room temperature, followed of appropriate anti-Ig horseradish peroxidase–conjugated polymer (Flex+; Dako). Sections were visualized with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with normal nonimmunized rabbit immunoglobulins were used as negative controls. As positive control, a section of colorectal tumor with known expression of SET was used. SET antibody sensitivity (1:5,000) had been calculated in a range of crescent dilutions of primary antibody. Specificity was confirmed in a set of paired fresh-frozen and FFPE samples were processed by Western blot analysis and IHC. Only the membrane of epithelial cells, but not stromal cells, was evaluated for SET expression blinded to clinical data by two pathologists (F. Rojo and S. Zazo). A semiquantitative histoscore was calculated by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low%) × 1 + (medium%) × 2 + (high%) × 3 and the results ranged from 0 to 300.

Statistical analysis

Statistical analyses were performed using SPSS 20 for windows (SPSS Inc.). OS was defined as the time from the date of surgery to the date of death from any cause or last follow-up. DFS was defined as the time from surgery until any primary, regional, or distant recurrence, appearance of a secondary tumor or death. Clinical benefit to oxaliplatin-based chemotherapy was defined as any response or prolonged stable disease (>12 weeks). The Kaplan–Meier method and survival comparisons were done with the log-rank test if proportional hazard assumption was fulfilled and Breslow otherwise. The Cox proportional hazards model was adjusted taking into consideration significant parameters in univariate analysis. A P value less than 0.05 was considered statistically significant. Receiver operating curve (ROC) was used to determine the optimal cutoff point based on progression endpoint for SET expression as previously described (35). This work was carried out in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines (36).

Results

SET is overexpressed and affects cell growth and colonosphere formation in colorectal cancer cells

We first confirmed SET deregulation by Western blot analysis in a set of primary colorectal cancer patients with paired normal mucosa and tumor samples, observing SET deregulated in 6 out of the 14 cases analyzed (Supplementary Fig. S1A). To investigate its biologic relevance as a potential oncogene in colorectal cancer, we assessed the effects of SET silencing on cell growth using two different specific siRNAs. Interestingly, we observed a decreased proliferation in SW480, HT-29, and LS513 cells transfected with any of the siRNAs against SET in comparison with cells transfected with a negative control siRNA (Fig. 1A). Of note, the effect in LS513 cells was less significant probably due to their lower SET expression levels. Similar results were obtained with the DLD-1, SW620, and HCT-116 cell lines (Supplementary Fig. S1B). In concordance with these results, SET silencing in SW480, HT-29, and LS513 cells led to a decreased colonosphere formation ability in both number (Fig. 1B) and size of colonospheres formed (Fig. 1C), indicating that SET is involved in colonosphere formation and self-renewal of colonosphere cells. These observations were confirmed in DLD-1, SW620, and HCT-116 cells (Supplementary Fig. S1C and S1D). Validation of SET silencing was performed by both real-time PCR and Western blot analysis (Supplementary Fig. S1E). Altogether, these results would indicate that SET overexpression is a common event that plays an oncogenic role in colorectal cancer.

SET overexpression leads to PP2A inhibition

Because SET has been reported as an endogenous PP2A inhibitor, we analyzed whether SET deregulation can alter the effects of the tumor-suppressor PP2A in colorectal cancer cells. Thus, we first confirmed by PP2A assays that ectopic expression of SET and PP2A leads to changes in the PP2A activity. As expected, we observed a PP2A inhibition after SET transfection, whereas SW480, HT-29, and LS513 cells transfected with PP2A showed increased PP2A activity levels. Moreover, overexpression of SET counteracted PP2A activation in cells ectopically expressing PP2A, and those cells showed PP2A activity levels similar to the corresponding controls (Fig. 2A). These observations were confirmed performing experiments with SET silencing (Supplementary Fig. S2A).

SET restores cell viability after PP2A overexpression

To analyze whether SET deregulation can alter the action of PP2A on cell growth, we next studied the effect of SET overexpression after PP2A activation. We observed an increased proliferation in SW480, HT-29, and LS513 cells transfected with SET in comparison with cells transfected with an empty vector (Fig. 2B). On the contrary, the activation of PP2A induced by its overexpression resulted in a decreased cell growth. Interestingly, we also observed that SET restored cell proliferation in SW480 cells ectopically expressing PP2A. Similar results were obtained in the HT-29 and LS513 cell lines (Fig. 2B). These observations were confirmed carrying out experiments with SET silencing (Supplementary Fig. S2B).

To further investigate the biologic effects of SET deregulation in colorectal cancer, we assessed apoptosis in SW480, HT-29, and LS513 cells ectopically expressing SET, PP2A, or both SET and PP2A. In concordance with its ability to impair cell proliferation, PP2A showed a caspase-dependent proapoptotic effect that was markedly reduced after SET overexpression (Supplementary Fig. S2C). Validation of SET overexpression was performed by both real-time PCR and

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

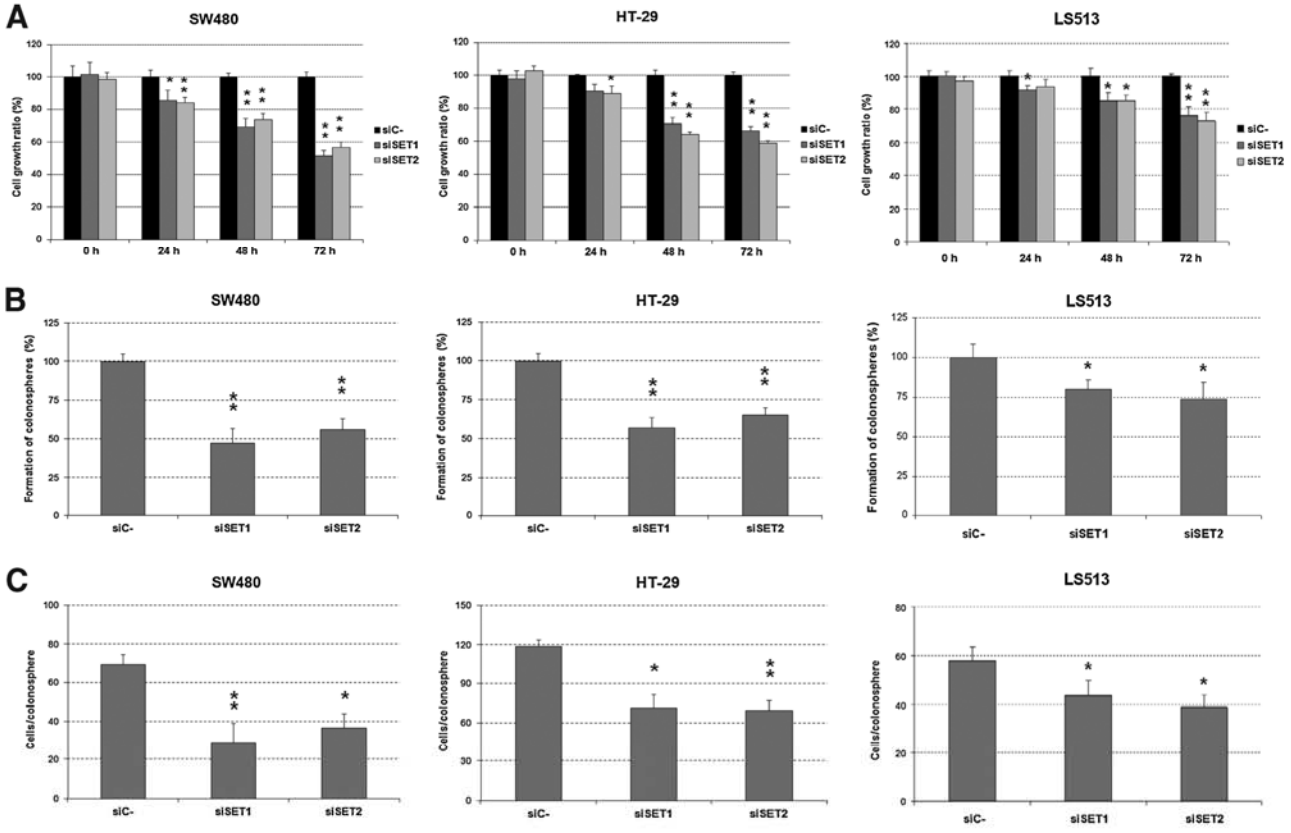

Figure 1.
SET proliferation and colonosphere formation in colorectal cancer cells. A, the MTS assay showing proliferation in SW480, HT-29, and LS513 cells after SET silencing. B, colonosphere formation and (C) evaluation of the number of cells per colonosphere after SET silencing; *, $P < 0.05$; **, $P < 0.01$.

Western blot analysis (Supplementary Fig. S2D). Thus, these results suggest that SET overexpression promotes cell proliferation and inhibits the antitumor effects of PP2A in colorectal cancer cells.

SET induces a decreased oxaliplatin sensitivity that is restored by FTY720 treatment

To assess a potential therapeutic role of SET affecting sensitivity of colorectal cancer cells to standard induction chemotherapy drugs used in this disease, we treated SW480 cells with oxaliplatin, alone or after transfection with a specific siRNA against SET. Interestingly, we observed that SET silencing enhanced sensitivity of SW480 cells to oxaliplatin treatment. These results were confirmed in the HT-29 and LS513 cell lines (Fig. 3A). When we examined 5-FU alone and in combination with oxaliplatin, higher sensitivity was also found after SET silencing (Supplementary Fig. S3A). Moreover, higher apoptosis was observed in SW480, HT-29, and LS513 cells treated with 5-FU combined with oxaliplatin when SET was silenced (Supplementary Fig. S3B). We also evaluated the sensitivity to oxaliplatin and 5-FU, observing a higher sensitivity to these drugs after SET silencing (Supplementary Fig. S3C). In concordance with these results, SW480, HT-29, and LS513 cells ectopically expressing SET showed an increased resistance to oxaliplatin. Interestingly, this effect was impaired when these cells were treated with oxaliplatin in combination with FTY720, a drug which showed marked antitumor properties (Fig. 3B). Effects of FTY720 alone or in combination with 5-FU or 5-FU and oxaliplatin were also evaluated observing similar results (Supplementary Fig. S4A and S4B). To further investigate the antitumor effects induced by the PP2A activation after FTY720 treatment or SET silencing, we carried out some cell-cycle analyses in SW480 and HT-29 cells, observing a reduction in S-phase together with an accumulation in G_0–G_1. As expected, these results were more evident after FTY720 treatment than after SET silencing (Supplementary Fig. S4C) probably because FTY720 acts via both SET and CIP2A inactivation (31, 32). Moreover, we observed lower sensitivity of colorectal cancer cell lines to oxaliplatin and 5-FU after ectopic expression of SET (Supplementary Fig. S4D). Therefore, these observations suggest that SET is involved in modulating sensitivity of colorectal cancer cells to oxaliplatin treatment, and that FTY720 impairs the SET-induced resistance to oxaliplatin.

Prevalence of SET overexpression in mCRC and its association with molecular and clinical parameters

To study the prevalence and clinical significance of SET overexpression, we quantified the expression of SET by immunohistochemistry in a cohort of 242 patients with mCRC. Patient characteristics are presented in Supplementary Table S1 and immunohistochemical detection of SET is shown in Supplementary Fig. S5. SET was overexpressed in 60 of 242 cases (24.8%). We found this alteration associated with worse

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

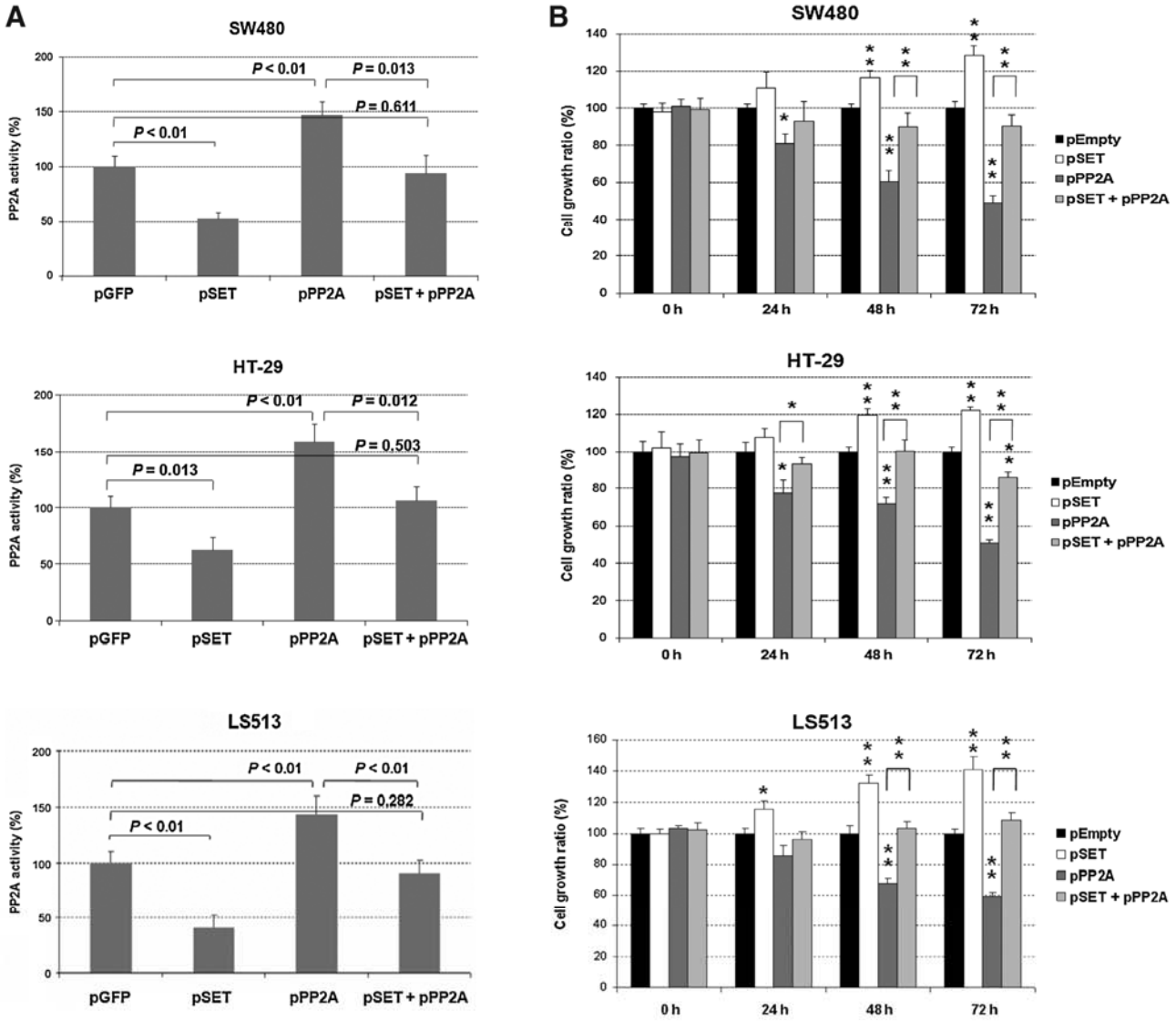

Figure 2.

SET inhibits PP2A and restores cell viability in colorectal cancer cells ectopically expressing PP2A. A, PP2A assay showing changes in PP2A activity after transfection with SET, PP2A, or with an empty vector; B, the MTS assay showing proliferation in SW480, HT-29, and LS513 cells transfected with PP2A alone or in combination with SET or with an empty vector; *, $P < 0.05$; **, $P < 0.01$.

Eastern Cooperative Oncology Group (ECOG) performance status (38.1% vs. 20%; $P = 0.013$), development of liver metastasis (29.3% vs. 15.4%; $P = 0.019$), and with the presence of synchronous metastasis at diagnosis (24.5% vs. 12.8%; $P = 0.018$). Moreover, the prevalence of SET overexpression was markedly higher in patients with colon primary tumors than in those with rectal primary tumors (32.1% vs. 11.8%; $P = 0.001$). Association between SET overexpression and molecular and clinical parameters are included in Table 1.

To further investigate the significance of SET deregulation in colorectal cancer, we next evaluated its expression in earlier stages of colorectal cancer, studying a cohort of 145 patients with colorectal cancer without metastatic disease. Interestingly, SET overexpression had a lower prevalence in this cohort (13.8%) compared with the mCRC cohort (24.8%). Moreover, we observed SET overexpression associated with those patients with rectal tumors although significance was not achieved ($P = 0.056$; Table 2).

Clinical significance of SET overexpression in mCRC

Clinical follow-up data were available for all the 242 patients with mCRC, 149 male and 93 female, with a median of age of 70 years (range, 29–93). The median OS of the global cohort was 21.9 months [95% confidence interval (CI), 17.2–26.5]. We found that the subgroup of patients with SET overexpressed showed a substantially shorter OS (median OS, 8.6 vs. 27 months; $P < 0.001$; Fig. 4A) and progression-free survival (PFS; median PFS, 7.1 vs. 13.7 months; $P < 0.001$; Fig. 4B). Whereas the prognostic impact of SET in OS was significant in both subgroups of patients younger (median OS, 10.2 vs. 34.6 months; $P < 0.001$) and older than 70 years (median OS, 6.5 vs. 18.1 months; $P = 0.015$), in PFS significance was only

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

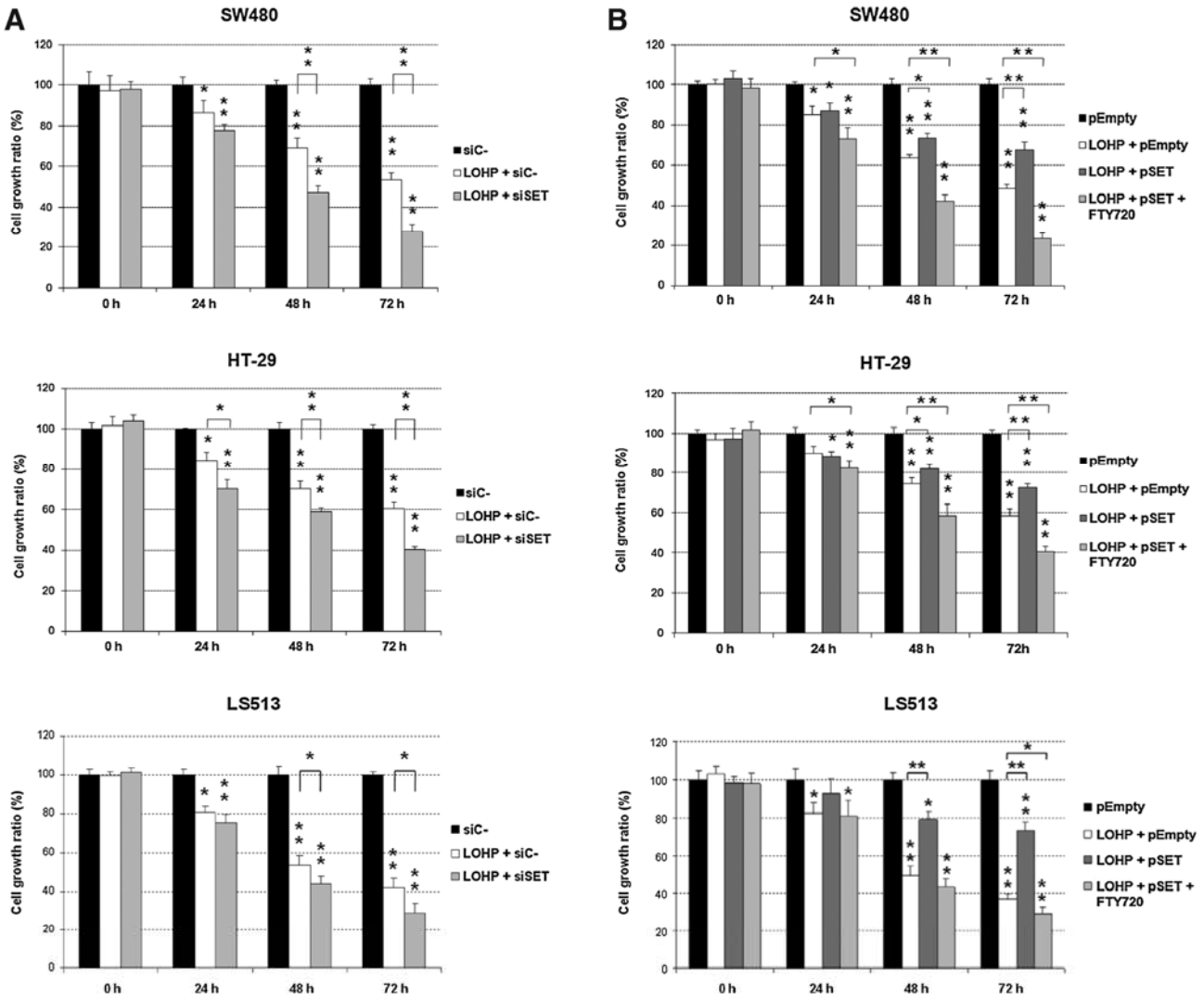

Figure 3.
SET induces a resistance of colorectal cancer cells to oxaliplatin that is impaired after FTY720 treatment. A, the MTS assays showing the effect of SET silencing in the sensitivity to oxaliplatin in SW480, HT-29, and LS513 cells. B, effects of FTY720 in SW480, HT-29, and LS513 cells ectopically expressing SET after oxaliplatin treatment; *, $P < 0.05$; **, $P < 0.01$.

achieved in the subgroup of younger patients (median PFS, 9.7 vs. 18.1 months; $P < 0.001$; Supplementary Fig. S5C). In addition, the prognostic impact of SET overexpression was independent of the KRAS mutation status, and was associated with shorter OS and PFS in both the KRAS wild-type (median OS, 9.7 vs. 25.3 months, $P = 0.009$; median PFS, 8.8 vs. 14.4 months, $P = 0.031$) and KRAS-mutated subgroups (median OS, 5.9 vs. 30.7 months, $P < 0.001$; median PFS, 4.5 vs. 12.5 months, $P = 0.002$; Supplementary Fig. S6A). Interestingly, we observed that SET overexpression was predictive of clinical benefit in those patients who received oxaliplatin-based chemotherapy ($N = 101$; $P = 0.004$; Supplementary Fig. S6B). Moreover, SET overexpression also determined worse OS (median OS, 17.4 vs. 34.6 months; $P < 0.001$) and PFS (median PFS, 11.3 vs. 14.5 months; $P = 0.024$) in this subgroup of cases (Fig. 4B). Of importance, multivariate analysis demonstrated that SET overexpression was an unfavorable independent factor associated with OS and PFS (Table 3) in mCRC. As indicated above, this study was performed in accordance with the REMARK guidelines.

Discussion

The SET oncogene has been reported to be upregulated in several types of cancer and it has been proposed as a candidate to develop novel molecular targeted therapies (21–23). Our previous results showed that PP2A inactivation is a common event in colorectal cancer and we identified SET deregulation as a possible contributing mechanism to inhibit PP2A in a set of 21 samples of patients with primary colorectal cancer (32). These results led us to hypothesize that SET could be playing an important oncogenic role in colorectal cancer. Although alterations affecting SET have been described in human cancer, its potential significance in colorectal cancer

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

Table 1. Association between SET and clinical and molecular parameters in 242 patients with mCRC

	Cases (*n*)	SET$^-$, *n* (%)	SET$^+$, *n* (%)	*P*
SET	242	182 (75.2)	60 (24.8)	
Sex	242	182	60	0.214
Male	149	108 (72.5)	41 (27.5)	
Female	93	74 (79.6)	19 (20.4)	
Age, y	230	174	56	0.875
<70	113	86 (76.1)	27 (23.9)	
≥70	117	88 (75.2)	29 (24.8)	
ECOG	222	170	52	**0.013**
0–2	180	144 (80)	36 (20)	
3–4	42	26 (61.9)	16 (38.1)	
Site of primary tumor	241	181	60	**0.001**
Colon	157	107 (68.2)	50 (31.8)	
Rectum	85	75 (88.2)	10 (11.8)	
Synchronous metastasis	242	182	60	**0.033**
No	84	70 (83.3)	14 (16.7)	
Yes	158	112 (70.9)	46 (28.1)	
Number of metastatic sites	242	182	60	0.980
1–2	218	164 (75.2)	54 (24.8)	
>2	24	18 (75)	6 (25)	
Liver metastasis	242	182	60	**0.019**
No	78	66 (84.6)	12 (15.4)	
Yes	164	116 (70.7)	48 (29.3)	
Lung metastasis	242	182	60	0.089
No	164	118 (72)	46 (28)	
Yes	78	64 (82.1)	14 (17.9)	
Lymph metastasis	242	182	60	0.378
No	176	135 (76.8)	41 (23.2)	
Yes	66	47 (71.2)	19 (28.8)	
Peritoneal metastasis	242	182	60	0.612
No	195	148 (75.9)	47 (24.1)	
Yes	47	34 (72.3)	13 (27.7)	
MSI	234	177	57	0.307
No	220	168 (76.4)	52 (23.6)	
Yes	14	9 (64.3)	5 (35.7)	
KRAS-mutated	238	180	58	0.571
No	136	101 (74.3)	35 (25.7)	
Yes	102	79 (77.5)	23 (22.5)	

remains unexplored. In this report, we show that SET is deregulated in colorectal cancer and plays an oncogenic role promoting cell proliferation and colonosphere formation, impairing PP2A antitumor activities, and modulating sensitivity of colorectal cancer cells to oxaliplatin treatment. Furthermore, our *in vitro* and clinical data provide evidences that SET overexpression is a recurrent alteration that predicts adverse outcome and induces a decreased sensibility to oxaliplatin in mCRC, which could be restored by the treatment with oxaliplatin in combination with FTY720.

Our previous findings showed that SET is overexpressed in colorectal cancer cell lines (32). To study the functional importance of SET deregulation, we silenced SET with two different specific siRNAs in five colorectal cancer cell lines and observed that in all cases SET silencing induced a decrease in the cell growth and colonosphere formation ability in both number and size of the spheres formed (Fig. 1 and Supplementary Fig. S1). These results indicate that SET deregulation is an alteration that plays a potential oncogenic role of SET in colorectal cancer.

Because SET deregulation has been reported as one of the molecular mechanisms that lead to PP2A inactivation (11, 17–18), we next analyzed the effects of SET regulating PP2A activity in colorectal cancer, and we observed that SET overexpression inhibits PP2A then restoring cell growth and decreasing apoptosis (Fig. 2 and Supplementary Fig. S2).

Our previous results showed SET deregulation in a short set of 21 patients with primary colorectal cancer but we did not analyze the molecular or clinical relevance of this finding. Therefore, we analyzed the potential significance of SET in a series of 242 cases with colorectal cancer stage IV, the subgroup of patients with colorectal cancer that represents the highest therapeutic challenge. In fact, the colorectal cancer stage IV is defined by the presence of metastatic disease and constitutes the subgroup of patients with the worst prognosis. Although the therapeutic advances implemented in the last years have affected the clinical outcome, unfortunately, their prognosis is still very poor. Therefore, it is necessary to develop alternative therapeutic strategies that improve the survival of these patients. Importantly, we observed that SET overexpression is a recurrent event (24.8%) that predicts adverse outcome and PFS in patients with mCRC (Fig. 4). In addition, we analyzed SET in a series of 145 patients with colorectal cancer without metastatic disease, observing a lower prevalence of SET overexpression in this cohort than in the metastatic cohort (13.8% vs 24.8%; Tables 1 and 2).

The high prevalence of this alteration suggests that SET overexpression is a key mechanism to inhibit PP2A in colorectal cancer cells, which could discriminate a subgroup of patients who might benefit from future therapies with PP2A activators. Furthermore, in concordance with the fact that SET silencing induced an increased sensitivity to oxaliplatin treatment in colorectal cancer cell lines (Fig. 3A), we observed that those patients without SET overexpression showed better response to oxaliplatin-based chemotherapy and longer OS and PFS (Fig. 4 and Supplementary Fig. S6B).

Table 2. Association between SET and clinical and molecular parameters in 145 colorectal cancer patients without metastatic disease

	Cases (*n*)	SET low, *n* (%)	SET high, *n* (%)	*P*
SET	145	125 (86.2)	20 (13.8)	
Sex	145	125	20	0.233
Male	87	77 (88.6)	10 (11.4)	
Female	58	48 (82.3)	10 (17.7)	
Age, y	145	125	20	0.077
<70	54	43 (79.6)	11 (20.4)	
≥70	91	82 (90.2)	9 (9.8)	
ECOG	145	125	20	0.876
0	69	59 (85.5)	10 (14.5)	
1	71	62 (87.3)	9 (12.7)	
2	5	4 (80)	1 (20)	
T	145	125	20	0.808
1	6	5 (83.3)	1 (16.7)	
2	31	28 (90.3)	3 (9.7)	
3	93	80 (86)	13 (14)	
4	15	12 (80)	3 (20)	
N	145	125	20	0.158
0	94	85 (90.4)	9 (9.6)	
1	32	26 (81.2)	6 (18.8)	
2	18	13 (72.2)	5 (27.8)	
x	1	1 (92.3)	0 (7.7)	
Site of primary tumor	142	122	20	0.056
Colon	109	97 (89)	12 (11)	
Rectum	33	25 (75.8)	8 (24.2)	

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

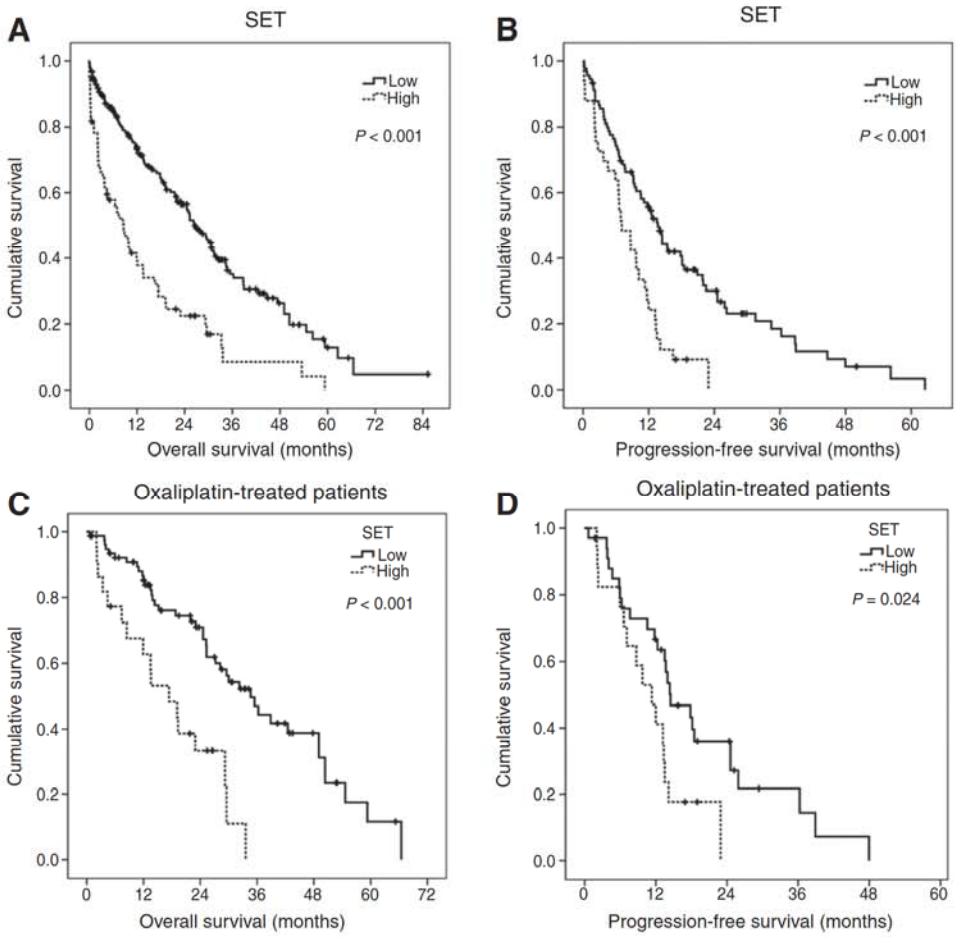

Figure 4.
The Kaplan–Meier analyses for SET in a cohort of 242 patients with mCRC: A, OS; B, PFS. The Kaplan–Meier analyses for SET in the subgroup of 101 patients with mCRC who received oxaliplatin-based chemotherapy: C, OS; D, PFS.

Interestingly, the observation that the PP2A activator FTY720, a drug with a promising preclinical antitumor efficacy in several cancer models including colorectal cancer (32, 37) that acts blocking SET (31), is able to impair the resistance induced by SET (Fig. 3B) would suggest that those patients with SET overexpression could improve their outcomes with a future inclusion of an oxaliplatin-based treatment in combination with FTY720 in the clinical protocols. In addition, the fact that the prognostic impact of SET was more evident in the subgroup of patients younger than 70 years (Supplementary Fig. S5C) is very interesting because this subgroup includes those cases susceptible of a more aggressive therapy that could benefit from the treatment with PP2A activators (e.g., FTY720).

The KRAS-mutated status is a very high prevalent alteration in mCRC that determines resistance to the monoclonal antibody cetuximab. Interestingly, it has recently reported that FTY720 could resensitize colorectal cancer cells to cetuximab, indicating a potential therapeutic relevance for FTY720 in mCRC (38). Of importance, when we stratify our series by the KRAS mutation status, the SET prognostic impact was particularly strong in those patients with KRAS-mutated ($P < 0.001$; Supplementary Fig. S6A). Our results would suggest that the presence of SET overexpression could cooperate inducing a higher resensitization to cetuximab after FTY720 treatment.

In conclusion, we show that SET overexpression is a recurrent molecular event that plays an oncogenic role in colorectal cancer contributing to inactivate the tumor-suppressor PP2A. Moreover, SET overexpression predicts worse outcome and response to oxaliplatin-based therapy, and its prognostic value is particularly significant in patients younger than 70 years and in those harboring KRAS mutations. Of importance, our results indicate that SET could serve to define a subgroup of patients with mCRC with worse outcome that could benefit by the future incorporation of PP2A-activating drugs, such as FTY720, in anticancer protocols.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: I. Cristóbal, C. Caramés, F. Rojo, J. García-Foncillas
Development of methodology: I. Cristóbal, R. Rincón, R. Manso, S. Zazo, F. Rojo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Cristóbal, R. Rincón, R. Manso, S. Zazo, F. Rojo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Cristóbal, R. Rincón, R. Manso, C. Caramés, F. Rojo, J. García-Foncillas
Writing, review, and/or revision of the manuscript: I. Cristóbal, R. Rincón, J. Madoz-Gúrpide, F. Rojo, J. García-Foncillas
Study supervision: I. Cristóbal, J. Madoz-Gúrpide, F. Rojo

Published OnlineFirst November 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0724

Table 3. Univariate and multivariate Cox analyses in the cohort of 242 patients with mCRC

	Univariate OS analysis		Multivariate OS Cox analysis	
	HR (95% CI, lower–upper)	**Significance**	**HR (95% CI, lower–upper)**	**Significance**
Age, y		**<0.001**		0.214
<70	1.000		1.000	
≥70	1.803 (1.301–2.499)		1.272 (0.871–1.857)	
Gender		0.475		—
Male	1.000			
Female	0.889 (0.645–1.226)		—	
Synchronous		0.112		—
No	1.000			
Yes	1.318 (0.938–1.852)		—	
ECOG		**<0.001**		**<0.001**
0–2	1.000		1.000	
3–4	1.906 (1.573–2.311)		1.706 (1.371–2.123)	
Number of metastatic sites		0.084		—
1–2	1.000			
>2	1.242 (0.971–1.590)		—	
SET		**<0.001**		**<0.001**
No	1.000		1.000	
Yes	2.490 (1.770–3.504)		2.097 (1.451–3.029)	
	Univariate PFS analysis		**Multivariate PFS analysis**	
	HR (95% CI, lower–upper)	**Significance**	**HR (95% CI, lower–upper)**	**Significance**
Age, y		**0.002**		0.066
	1.000		1.000	
	1.852 (1.243–2.760)		1.533 (0.972–2.417)	
Gender		0.595		—
Male	1.000			
Female	0.897 (0.601–1.339)		—	
Synchronous		0.082		—
No	1.000			
Yes	1.473 (0.952–2.281)		—	
ECOG		**<0.001**		**0.023**
0–2	1.000		1.000	
3–4	1.556 (1.224–1.978)		1.369 (1.045–1.795)	
Number of metastatic sites		**0.029**		**0.004**
1–2	1.000		1.000	
>2	1.374 (1.033–1.827)		1.547 (1.154–2.074)	
SET		**<0.001**		**0.001**
No	1.000		1.000	
Yes	2.323 (1.486–3.633)		2.192 (1.357–3.541)	

Abbreviation: HR, hazard ratio. Statistically significant values are in bold.

Acknowledgments

The authors thank Cristina Chamizo for technical assistance.

Grant Support

This study was supported by the Biobank of "Fundación Jiménez Díaz" (FJD Biobank; RD12/0036/0021), grants from "Instituto de Salud Carlos III FEDER" (PI12/01552 and PI13/02609), and S2010/BMD2344. R. Manso is supported by "Fundación Conchita Rábago de Jiménez Díaz."

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Received March 25, 2014; revised October 30, 2014; accepted November 4, 2014; published OnlineFirst November 11, 2014.

References

1. El Zouhairi M, Charabaty A, Pishvaian MJ. Molecularly targeted therapy for metastatic colon cancer: proven treatments and promising new agents. Gastrointest Cancer Res 2011;4:15–21.
2. Lermos C, Sack U, Schmid F, Juneja M, Stein U. Anti-metastatic treatment in colorectal cancer: targeting signaling pathways. Curr Pharm Des 2013;19:841–63.
3. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J Biol Chem 1996;271:11059–62.
4. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell 2001;104:119–30.
5. Kandilci A, Mientjes E, Grosveld G. Effects of SET and SET-CAN on the differentiation of the human promonocytic cell line U937. Leukemia 2004;18:337–40.
6. ten Klooster JP, Leeuwen I, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET. Embo J 2007;26:336–45.
7. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, Diaz C, Pujol MJ, Agell N, et al. The SET protein regulates G_2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. J Biol Chem 2003;278:1158–64.
8. Trotta R, Ciarlariello D, Dal Col J, Allard J II, Neviani P, Santhanam R, et al. The PP2A inhibitor SET regulates natural killer cell IFN-gamma production. J Exp Med 2007;204:2397–2405.
9. Trotta R, Ciarlariello D, Dal Col J, Mao H, Chen L, Briercheck E, et al. The PP2A inhibitor SET regulates granzyme B expression in human natural killer cells. Blood 2011;117:2378–84.
10. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active

demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem 2002;277:25026–31.
11. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med 2008;14:152–60.
12. Al-Murrani SW, Woodgett JR, Damuni Z. Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. Biochem J 1999;341:293–8.
13. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell 2003;112:659–72.
14. Kim JY, Lee KS, Seol JE, Yu K, Chakravarti D, Seo SB. Inhibition of p53 acetylation by INHAT subunit SET/TAF-Iβ represses p53 activity. Nucleic Acids Res 2012;40:75–87.
15. Liu GP, Wei W, Zhou X, Zhang Y, Shi HH, Yin J, et al. I(2)(PP2A) regulates p53 and Akt correlatively and leads the neurons to abort apoptosis. Neurobiol Aging 2012;33:254–64.
16. Leopoldino AM, Squarize CH, Garcia CB, Almeida LO, Pestana CR, Polizello AC, et al. Accumulation of the SET protein in HEK293T cells and mild oxidative stress: cell survival or death signaling. Mol Cell Biochem 2012;363:65–74.
17. Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW, Liu S, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell 2005;8:355–68.
18. Cristobal I, Garcia-Orti L, Cirauqui C, Cortes-Lavaud X, Garcia-Sanchez MA, Calasanz MJ, et al. Overexpression of SET is a recurrent event associated with poor outcome and contributes to protein phosphatase 2A inhibition in acute myeloid leukemia. Haematologica 2012;97:543–50.
19. Sirma Ekmekci S, G Ekmekci C, Kandilci A, Gulec C, Akbiyik M, Emrence Z, et al. SET oncogene is upregulated in pediatric acute lymphoblastic leukemia. Tumori 2012;98:252–6.
20. Leopoldino AM, Squarize CH, Garcia CB, Almeida LO, Pestana CR, Sobral LM, et al. SET protein accumulates in HNSCC and contributes to cell survival: antioxidant defense, Akt phosphorylation and AVOs acidification. Oral Oncol 2012;48:1106–13.
21. Christensen DJ, Chen Y, Oddo J, Matta KM, Neil J, Davis ED, et al. SET oncoprotein overexpression in B-cell chronic lymphocytic leukemia and non-Hodgkin lymphoma: a predictor of aggressive disease and a new treatment target. Blood 2011;118:4150–8.
22. Switzer CH, Cheng RYS, Vitek TM, Christensen DJ, Wink DA, Vitek MP. Targeting SET/I2PP2A oncoprotein functions as a multi-pathway strategy for cancer therapy. Oncogene 2011;30:2504–13.
23. Mukhopadhyay A, Tabanor K, Chaguturu R, Aldrich JV. Targeting inhibitor 2 of protein phosphatase 2A as a therapeutic strategy for prostate cancer treatment. Cancer Biol Ther 2013;14:962–72.
24. Chao A, Tsai CL, Wei PC, Hsueh S, Chao AS, Wang CJ, et al. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. Cancer Lett 2010;291:99–107.
25. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007;130:21–4.
26. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. Curr Opin Genet Dev 2005;15:34–41.
27. Eichhorn PJ, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. Biochim Biophys Acta 2009;1795:1–15.
28. Cristobal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD. PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. Leukemia 2011;25:606–14.
29. Kalev P, Sablina AA. Protein phosphatase 2A as a potential target for anticancer therapy. Anticancer Agents Med Chem 2011;11:38–46.
30. Perrotti D, Neviani P. Protein phosphatise 2A: a target for anticancer therapy. Lancet Oncol 2013;14:e229–38.
31. Saddoughi SA, Gencer S, Peterson YK, Ward KE, Mukhopadhyay A, Oaks J, et al. Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumor suppression via activation of PP2A-RIPK1-dependent necroptosis. EMBO Mol Med 2013;5:105–21.
32. Cristobal I, Manso R, Rincon R, Carames C, Senin C, Borrero A, et al. PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. Mol Cancer Ther 2014;13:938–47.
33. Bitarte N, Bandres E, Boni V, Zarate R, Rodríguez J, Gonzalez-Huarriz M, et al. MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. Stem Cells 2011;29:1661–71.
34. Cristobal I, Rincon R, Manso R, Madoz-Gurpide J, Carames C, del Puerto-Nevado L, et al. Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation. Biochim Biophys Acta 2014;1842:1823–9.
35. Generali D, Buffa FM, Berruti A, Brizzi MP, Campo L, Bonardi S , et al. Phosphorylated ERalpha, HIF-1alpha, and MAPK signaling as predictors of primary endocrine treatment response and 470 resistance in patients with breast cancer. J Clin Oncol 2009;27:227–34.
36. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clarck GM. Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 2005;23:9067–72.
37. Zhang L, Wang HD, Ji XJ, Cong ZX, Zhu JH, Zhou Y. FTY720 for cancer therapy (review). Oncol Rep 2013;30:2571–8.
38. Rosa R, Marciano R, Malapelle U, Formisano L, Nappi L, D'Amato C, et al. Sphingosine kinase 1 overexpression contributes to cetuximab resistance in human colorectal cancer models. Clin Cancer Res 2013;19:138–47.

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Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer

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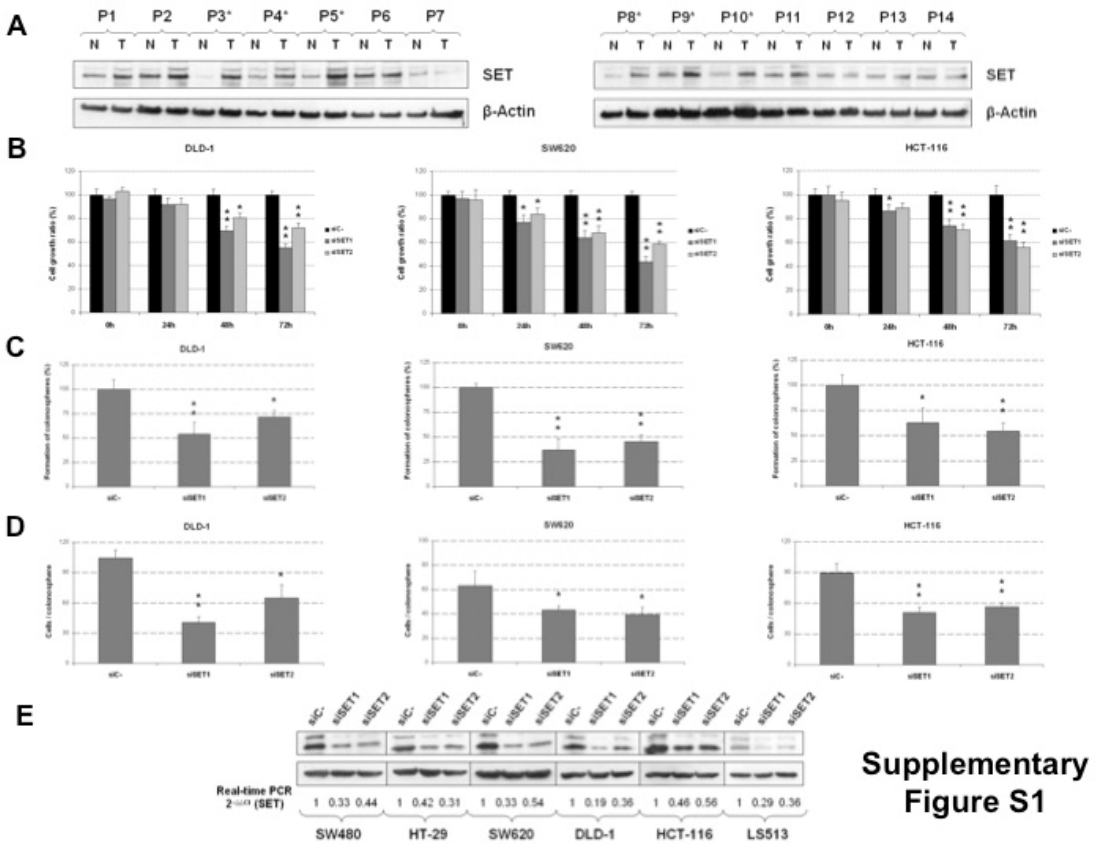
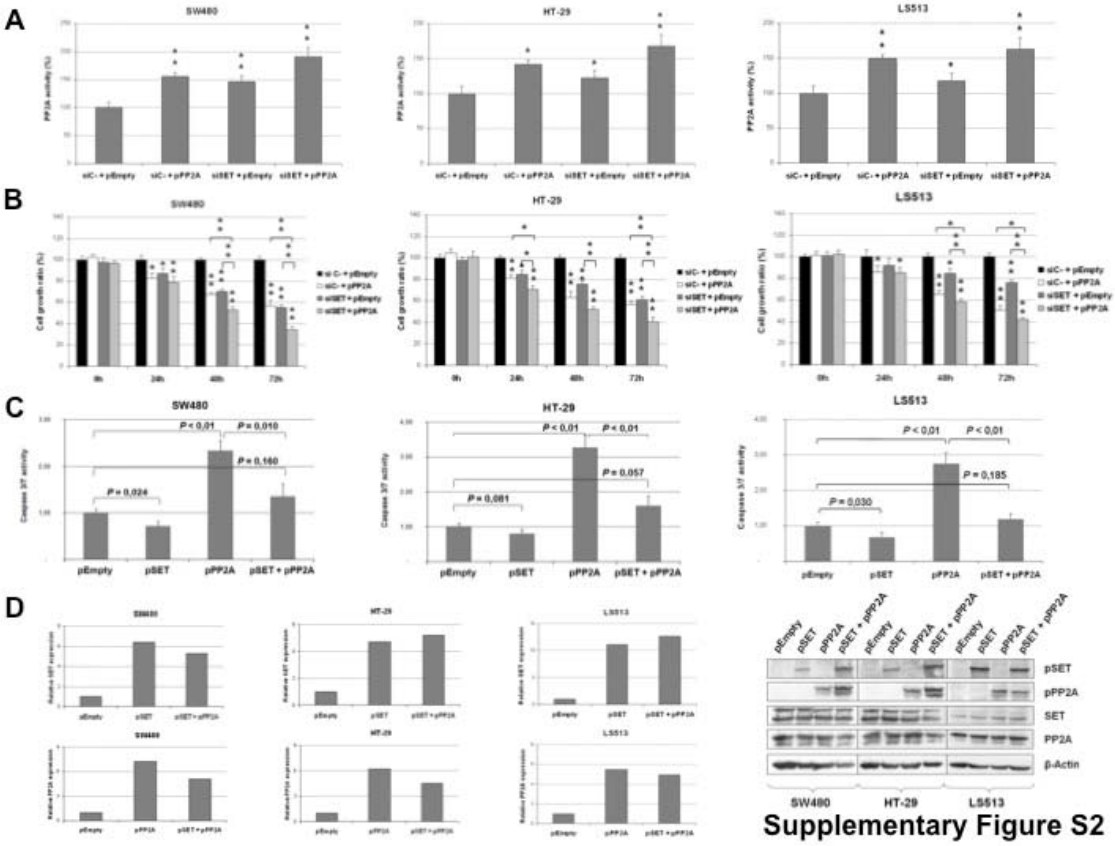
Clin Cancer Res 2015;21:347-356. Published OnlineFirst November 11, 2014.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. SET is deregulated in CRC patients and induces proliferation and colonosphere formation in DLD-1, SW620 and HCT-116 cells. (A) Western blot analysis of SET expression in CRC patients with paired normal colonic mucosa and tumor samples; * SET overexpression; N: normal mucosa; T: tumor sample. (B) MTS assays showing proliferation in CRC cells after SET silencing. (C) Colonosphere formation and (D) evaluation of the number of cells per colonosphere after SET silencing. (E) Validation of the SET silencing by real-time PCR and western blot; * $P < 0.05$; ** $P < 0.01$.

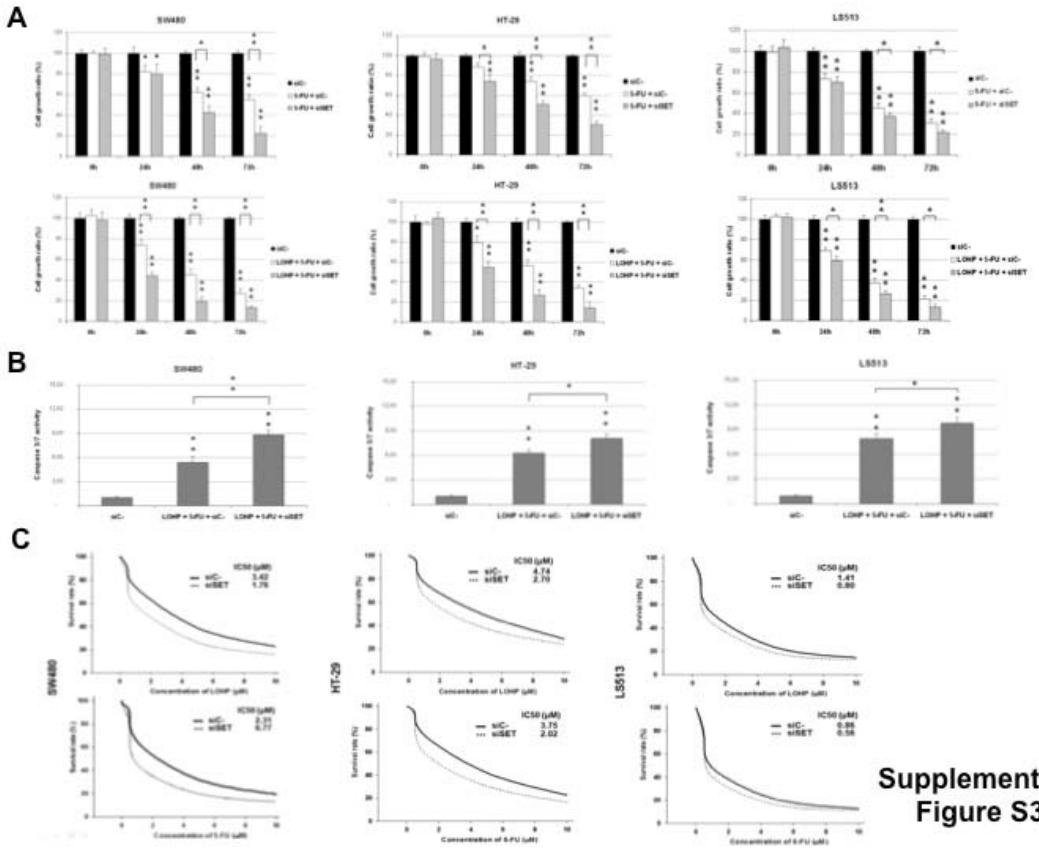


Supplementary Figure S2. SET modulation in CRC cells ectopically expressing PP2A. (A) PP2A and (B) MTS assays after SET silencing. (C) Caspase 3/7 assays in SW480, HT-29 and LS513 cells after transfection with SET, PP2A or with both SET and PP2A. (D) Validation of SET overexpression by real time PCR (2-ΔΔCt) and western blot; * P < 0.05; ** P < 0.01.



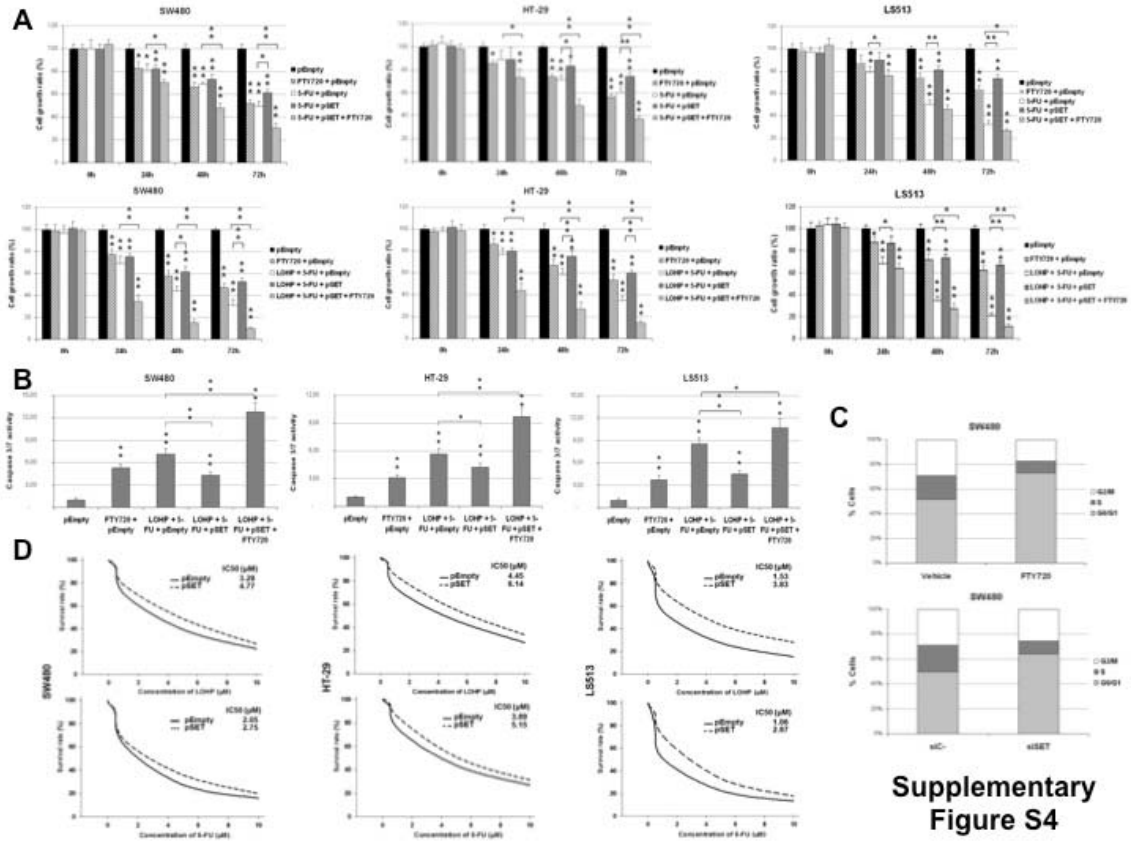
Supplementary Figure S2

Supplementary Figure S3. Effect of SET silencing in the sensitivity of CRC cells to 5-FU alone or combined with oxaliplatin. (A) MTS, (B) caspase 3/7 assays and (C) drug concentration curves to evaluate the effect of SET silencing in the sensitivity to oxaliplatin and 5-FU in SW480, HT-29 and LS513 cells; * P < 0.05; ** P < 0.01.

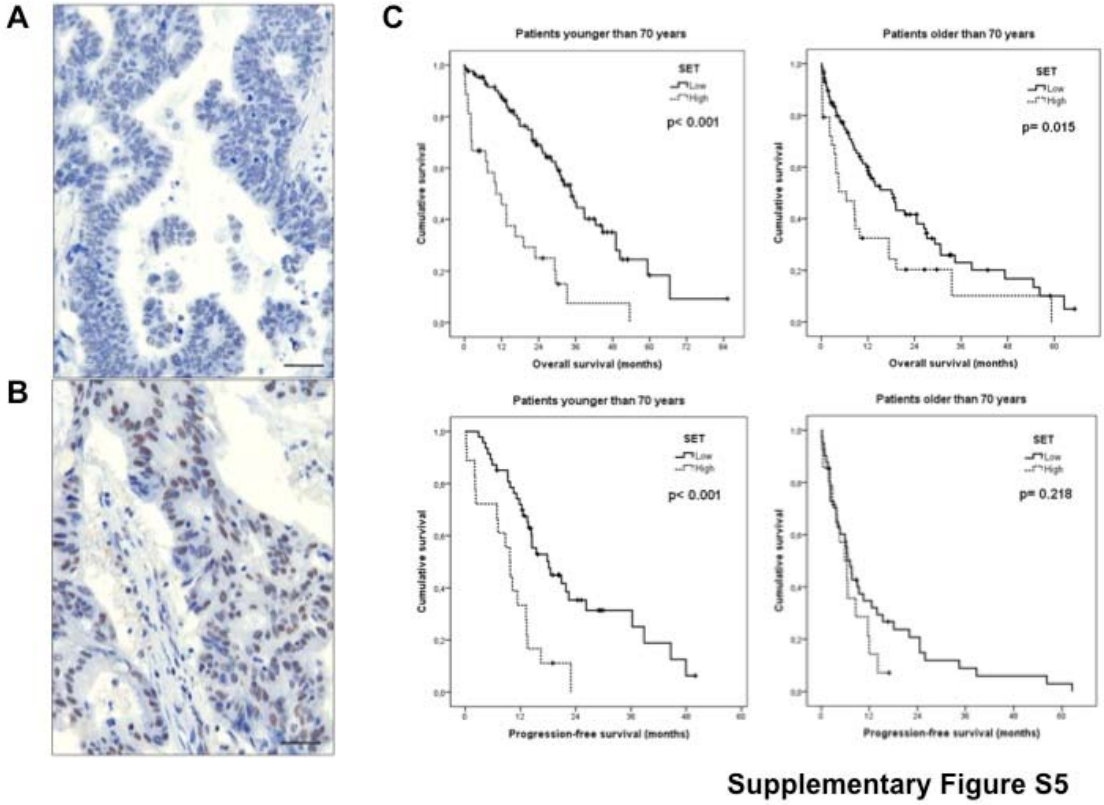


Supplementary Figure S3

Supplementary Figure S4. Effects of FTY720 in SW480, HT-29 and LS513 cells ectopically expressing SET and treated with 5-FU alone or combined with oxaliplatin. (A) MTS, (B) caspase 3/7 assays, (C) cell cycle analysis and (D) drug concentration curves to evaluate the effect of SET overexpression in the sensitivity to oxaliplatin and 5-FU in SW480, HT-29 and LS513 cells; * P < 0.05; ** P < 0.01.

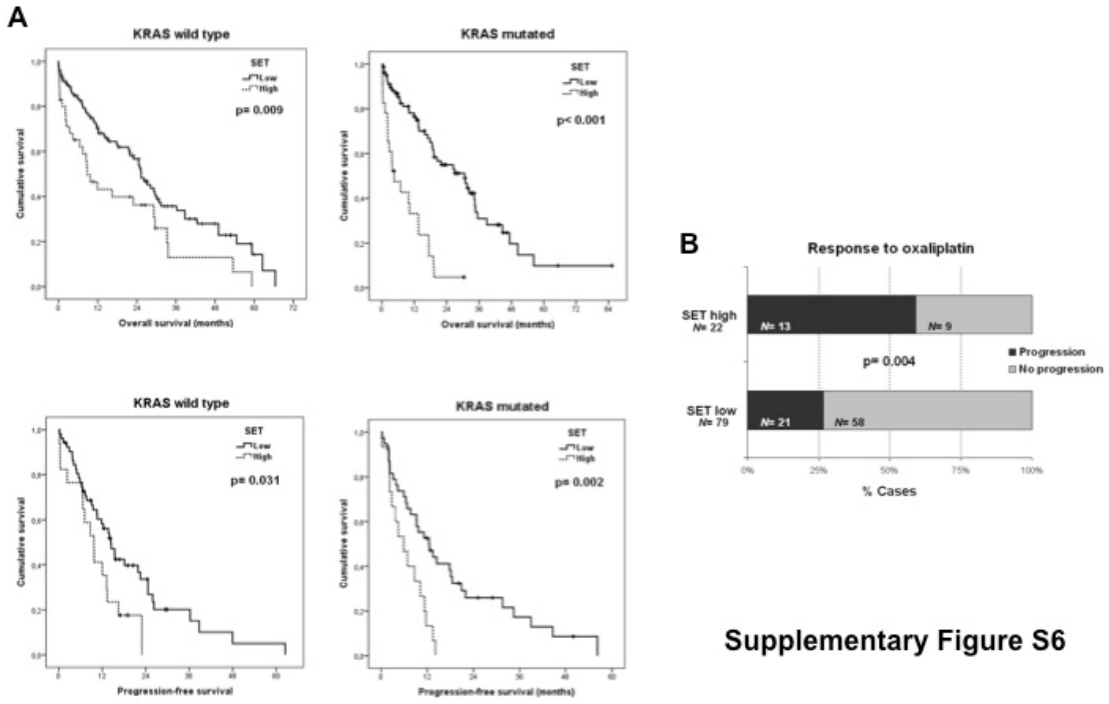


Supplementary Figure S5. Immunohistochemical detection of SET expression in patients with metastatic colorectal cancer showing SET negative (A) and positive (B) stainings. The line in A and B shows 25 µm. Magnification x400. (C) Kaplan-Meier analyses of overall and progression-free survival and in the subgroups of patients younger (N = 113) and older than 70 years (N = 117).



Supplementary Figure S5

Supplementary Figure S6. (A) Kaplan-Meier analyses of overall and progression-free survival and in the subgroups of patients with KRAS wild type (N = 136) and KRAS mutated (N = 102). (B) Predictive value of response to treatment by SET deregulation in those patients who received oxaliplatin-based chemotherapy (N= 101).



Supplementary Figure S6

Supplementary Table S1. Clinical and molecular characteristics of a series of 242 patients with metastatic CRC.

		No. (%)
Sex		
	Male	149 (61.6)
	Female	93 (38.4)
Age		
	<70	113 (49.1)
	≥70	117 (50.9)
	No data	12
ECOG		
	0-2	180 (81.1)
	3-4	42 (18.9)
	No data	20
MSI		
	No	220 (94)
	Yes	14 (6)
	No data	8
KRAS mutations		
	No	136 (57.1)
	Yes	102 (42.9)
	No data	4
Site of primary tumor		
	Colon	157 (64.9)
	Rectum	85 (35.1)
Synchronous metastasis		
	No	84 (34.7)
	Yes	158 (65.3)
Number of metastatic sites		
	1-2	218 (90.1)
	>2	24 (9.9)
Liver metastasis		
	No	78 (32.2)
	Yes	164 (67.8)
Lung metastasis		
	No	164 (67.8)
	Yes	78 (32.2)
Lymph metastasis*		
	No	176 (72.7)
	Yes	66 (27.3)
Peritoneal metastasis		
	No	195 (80.6)
	Yes	47 (19.4)
Prior adjuvant chemotherapy**		
	No	32 (38.1)
	Yes	52 (61.9)
Treatment 1st line metastatic		
	Oxaliplatin	101 (43.2)
	Irinotecan	38 (16.2)
	5-FU	22 (9.4)
	None	73 (31.2)
	No data	8

*Non-regional lymph node involvement; **Cases with metachronous metastasis only

Article 5: Downregulation of microRNA 199b predicts unfavorable prognosis and emerges as a novel therapeutic target which contributes to PP2A inhibition in metastatic colorectal cancer.[5]

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Downregulation of microRNA-199b predicts unfavorable prognosis and emerges as a novel therapeutic target which contributes to PP2A inhibition in metastatic colorectal cancer

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Keywords: *miR-199b, SET, PP2A, prognosis, therapy*

Received: *November 02, 2015* ***Accepted:*** *July 17, 2016* ***Published:*** *August 10, 2016*

ABSTRACT

The tumor suppressor microRNA-199b (miR-199b) is a negative SET regulator associated with poor outcome in some human cancers. However, its expression levels as well as potential biological and clinical significance in colorectal cancer (CRC) remain completely unexplored. The PP2A inhibitor SET has shown promising therapeutic and clinical implications in metastatic CRC (mCRC) but the molecular mechanisms underlying SET deregulation are currently unknown. We show here miR-199b downregulation in 4 out of 5 CRC SET-overexpressing cell lines and its inverse correlation with SET overexpression in CRC patients. Moreover, miR-199b led to PP2A activation through a direct SET inhibition, impaired cell viability and enhanced oxaliplatin sensitivity in CRC cells. MiR-199b was found downregulated in 25% of cases, and associated with lymph metastasis ($p = 0.049$), presence of synchronous metastasis at diagnosis ($p = 0.026$) and SET overexpression ($p < 0.001$). Furthermore, low miR-199b levels determined shorter overall ($p < 0.001$), progression-free survival ($p = 0.003$) and predicted clinical benefit to oxaliplatin treatment. The miR-199b prognostic impact was particularly evident in both younger and *KRAS* wild-type subgroups. Multivariate analyses confirmed its independent prognostic impact. Altogether, our results show that miR-199b is a tumor suppressor whose downregulation independently determines worse outcome and emerges as a potential contributing mechanism to inhibit PP2A via SET overexpression in a subgroup of mCRC patients.

INTRODUCTION

Colorectal cancer (CRC) represents the third most commonly diagnosed cancer and the fourth highest cause of cancer-related deaths worldwide [1]. Altough the CRC stage at diagnosis is the most predictive factor of clinical outcome, more than 70% of CRC cases newly diagnosed have a surgically resectable localized disease [2]. However, the remaining 20–30% of newly diagnosed CRC patients with unresectable distant metastasis together with a substantial proportion of cases who develop metachronous metastasis represents the subgroup of patients with worst outcome [3]. Therefore, it remains necessary to improve our molecular knowledge of CRC to identify alterations with both prognostic and predictive value of therapy efficacy to develop novel and more efficient targeted therapies.

The protein phosphatase 2A (PP2A) is a well-known tumor suppressor that inhibits signaling pathways critical in human cancer [4, 5]. Several works highlighting the

molecular and clinical significance of PP2A inhibition in CRC have been reported [6–9]. The protein SET is a potent endogenous PP2A inhibitor [10] involved in many cell functions [11–15] and a novel proposed target for anticancer therapy [16]. Interestingly, some evidences suggest that SET could be relevant in CRC progression [17]. In fact, our group has recently reported that SET deregulation determines poor outcome and defines a subgroup of metastatic CRC patients who could benefit from therapies containing PP2A activators [18]. MicroRNAs (miRs) are small non-coding RNAs that inhibit specific target genes by translation repression and they then can function as oncogenes or tumor suppresors in human cancer. MiR-199b is a SET inhibitor [19, 20] which has also been involved in acquired chemoresistance in chronic myeloid leukaemia or ovarian cancer [21, 22]. Moreover, miR-199b also functions as a tumor suppressor in medulloblastoma, hepatocellular carcinoma and breast cancers by affecting targets such as HEIS1, HIF1α or HER2 [23–25]. However, its status and potential significance in colorectal cancer is completely unknown.

In this report, we identified miR-199b downregulation as a common alteration with high clinical relevance that represents a potential contributing mechanism to SET overexpression in metastatic CRC patients. Interestingly, low miR-199b levels inversely correlated with SET expression and independently predicted shorter overall and progression-free survival defining a subgroup of metastatic CRC patients with very poor outcome candidate to be treated with SET/PP2A targeting drugs such as FTY720.

RESULTS

MiR-199b is downregulated and affects SET expression and PP2A activation status in CRC cells

We quantified miR-199b in 5 different CRC cell lines, observing low miR-199b levels in 4 out of the 5 CRC cell lines compared to normal colonic mucosa (Supplementary Table S1). Moreover, western blot analysis showed SET overexpression in the same 5 CRC cell lines (Figure 1A). The same normal controls were used in both experiments. We first performed luciferase assays to validate the role of miR-199b as a negative SET regulator in CRC. Transfection of pSET-3′UTRwt in SW480 cells ectopically expressing miR-199b showed decreased luciferase activity, indicating that miR-199b binds to the SET 3′UTR, negatively regulating its expression. Analysis using the same construct with the mutated miR-199b seed region showed no changes in luciferase activity, confirming that miR-199b directly binds to SET (Supplementary Figure S1). We next assessed the effects of miR-199b modulation on SET expression in SW480 cells using pre- and anti-microRNAs specific for miR-199b. As expected, we found decreased and increased SET levels in SW480 cells transfected with pre- and anti-miR-199b, respectively (Figure 1B). Similar results were obtained using HT-29 cells (Supplementary Figure S2A).

Due to SET is an endogenous PP2A inhibitor and miR-199b negatively regulates SET, we analyzed whether miR-199b deregulation could modulate PP2A in CRC cells. As expected, we observed PP2A activation in both SW480 and HT-29 cells after pre-miR-199b transfection. Although transfection with anti-miR-199b induced PP2A inhibition significance was only achieved in HT-29 cells. (Figure 1C and Supplementary Figure S2B). These results prompted us to analyze SET and miR-199b expression levels in a cohort of 97 patients with metastatic CRC. Patient characteristics are presented in Supplementary Table S2. Interestingly, a negative correlation was found between miR-199b and SET expression (Supplementary Figure S3). Moreover, significant lower miR-199b was significantly downregulated in the subgroup of patients with SET overexpression (Figure 1D), suggesting that altered expression miR-199b is a molecular mechanism that contributes to deregulate SET and PP2A activation status in CRC patients.

MiR-199b impairs cell viability in a SET-dependent manner

To investigate its biological relevance as a potential tumor suppressor in CRC, we assessed the effects of miR-199b modulation on cell growth. Interestingly, we observed a reduced proliferation in SW480 cells transfected with a pre-miR-199b in comparison with those transfected with a negative control (Figure 2A). These results were confirmed with the HT-29 cell line (Supplementary Figure S4A). However, only slight effects on cell growth were found by anti-miR-199b in SW480 and HT-29 cells (Figure 2B and Supplementary Figure S4B). Of importance, we also observed that ectopic expression of SET significantly restored cell proliferation in SW480 cells transfected with pre-miR-199b (Figure 2C). Similar results were found in HT-29 cells (Supplementary Figure S4C). Altogether, these results would indicate that SET regulation is a key event which mediates miR-199b-induced antitumor effects in CRC.

MiR-199b sensitizes CRC cells to oxaliplatin and 5-FU treatments

We next investigated the potential therapeutic role of miR-199b affecting sensitivity of CRC cells to standard chemotherapy drugs such as oxaliplatin and 5-FU. Interestingly, we found that miR-199b-overexpressing SW480 cells showed higher sensitivity to oxaliplatin treatment. These results were confirmed in the HT-29 cell line (Figure 3A). Similarly, we observed

an enhanced sensitivity to 5-FU treatment in both SW480 and HT-29 cells transfected with pre-miR-199b (Figure 3B). In order to assess whether miR-199b affects oxaliplatin sensitivity through SET inhibition, we modulated SET expression in oxaliplatin treated SW480 and HT-29 cells ectopically expressing miR-199b. Interestingly, we observed that SET overexpression was able to restore oxaliplatin sensitivity (Supplementary Figure S5), suggesting that miR-199b regulates oxaliplatin sensitivity in CRC cells through a SET negative regulation.

Prevalence of miR-199b downregulation in metastatic colorectal cancer and its association with molecular and clinical parameters

In order to investigate whether miR-199b is deregulated in CRC patients, we analyzed miR-199b expression levels in a cohort of 97 patients with metastatic CRC. Mir-199b was found downregulated in 24 of 97 cases (24.7%). Patient characteristics are presented in Table S2. Interestingly, we found low miR-199b expression associated with development of lymph metastasis (37.9% versus 19.1%, $p = 0.049$), presence of synchronous metastasis at diagnosis (32.8% versus 12.8%, $p = 0.026$) and SET overexpression (53.1% versus 10.8%, $p < 0.001$). Association between miR-199b downregulation and molecular and clinical parameters are included in Table 1. Interestingly, we observed miR-199b dowregulated in 17 out of 32 cases with SET overexpression, suggesting that low miR-199 is a relevant contributing alteration to deregulate SET in a subgroup of CRC patients. Of importance, these findings would also indicate the existence of alternative mechanisms in those SET-overexpressing cases without miR-199b downregulation that should have to be elucidated in future studies.

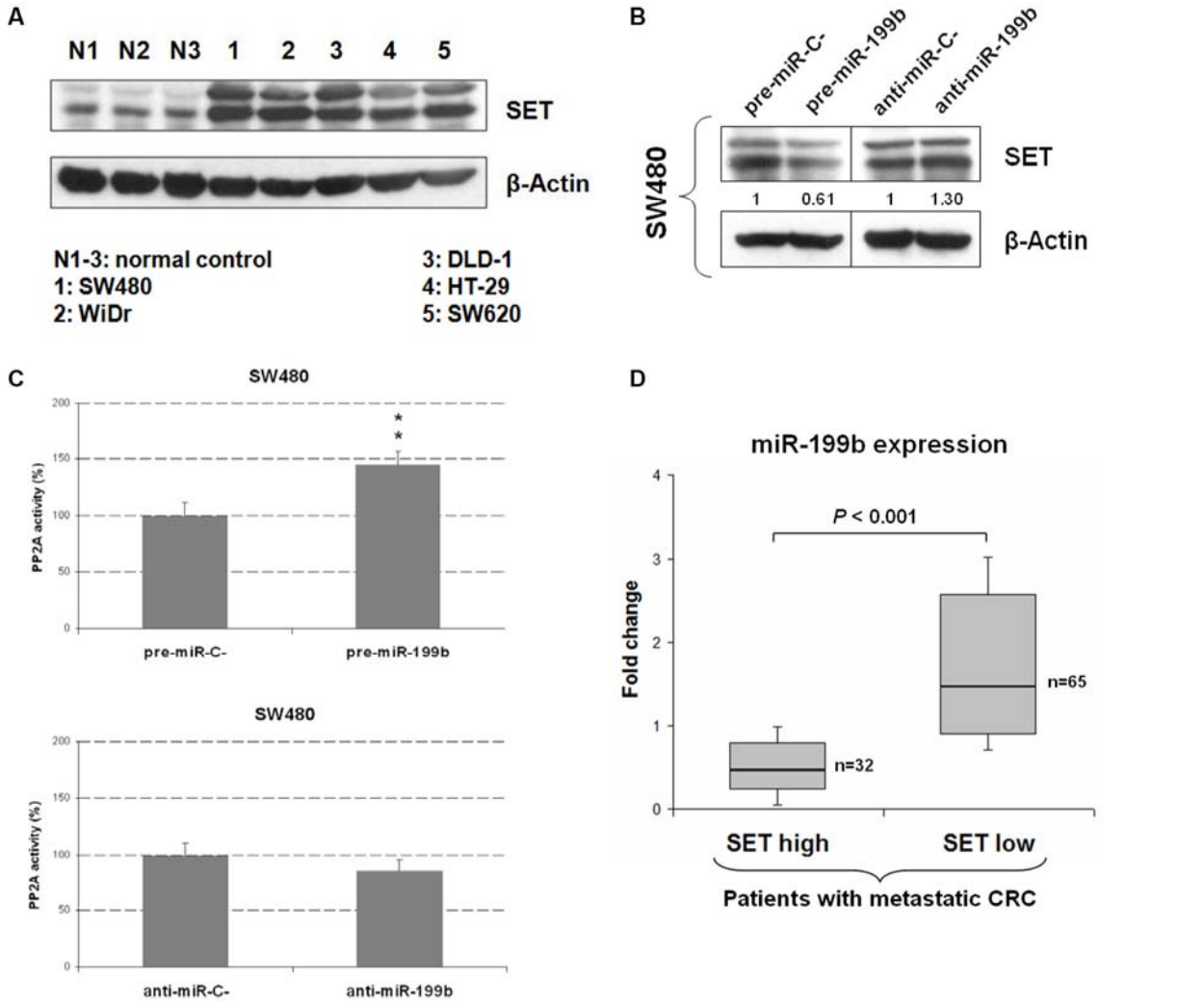


Figure 1: MiR-199b downregulation is a common alteration in mCRC that regulates SET and PP2A activity (A) Western blot analysis showing SET expression levels in 5 CRC cell lines. (**B**) Western blot analysis showing SET expression in SW480 cells transfected with pre- or anti-miR-199b; (**C**) PP2A assays showing changes in PP2A activity in SW480 cells after transfection with pre- or anti-miR-199b. Data represented are mean of three independent experiments ± SD. *$P < 0.05$; **$P < 0.01$; (**D**) Box-plot showing miR-199b expression levels in patients with ($N = 35$) and without ($N = 62$) SET overexpression; N1-3: normal controls.

Table 1: Association between miR-199b and clinical and molecular parameters in 97 patients with metastatic CRC

	No. Cases	High miR-199b (%)	Low miR-199b (%)	*P*
miR-199b	97	73 (75.3)	24 (24.7)	
Sex	97	73	24	0.830
Male	67	50 (74.6)	17 (25.4)	
Female	30	23 (76.7)	7 (23.3)	
Age	93	71	22	0.079
< 70	44	30 (68.2)	14 (31.8)	
≥ 70	49	41 (83.7)	8 (16.3)	
ECOG	92	71	21	0.175
0–2	75	60 (80)	15 (20)	
3–4	17	11 (64.7)	6 (35.3)	
Site of primary tumor	97	73	24	0.524
Colon	72	53 (73.6)	19 (26.4)	
Rectum	25	20 (80)	5 (20)	
Synchronous metastasis	97	73	24	**0.026**
No	39	34 (87.2)	5 (12.8)	
Yes	58	39 (67.2)	19 (32.8)	
Number of metastatic sites	97	73	24	0.383
1–2	89	68 (76.4)	21 (23.6)	
> 2	8	5 (62.5)	3 (37.5)	
Liver metastasis	97	73	24	0.282
No	33	27 (81.8)	6 (18.2)	
Yes	64	46 (71.9)	18 (28.1)	
Lung metastasis	97	73	24	0.264
No	68	49 (84.5)	19 (15.5)	
Yes	29	24 (82.8)	5 (17.2)	
Lymph metastasis	97	73	24	**0.049**
No	68	55 (80.9)	13 (19.1)	
Yes	29	18 (62.1)	11 (37.9)	
Peritoneal metastasis	97	73	24	0.173
No	78	61 (78.2)	17 (21.8)	
Yes	19	12 (63.2)	7 (36.8)	
MSI	95	71	24	0.617
No	89	66 (74.2)	23 (25.8)	
Yes	6	5 (83.3)	1 (16.7)	
KRAS mutated	97	73	24	0.755
No	58	43 (74.1)	15 (25.9)	
Yes	39	30 (76.9)	9 (23.1)	
SET overexpression	97	73	24	**< 0.001**
No	65	58 (89.2)	7 (10.8)	
Yes	32	15 (46.9)	17(53.1)	

Table 2: Univariate and multivariate Cox analyses in the cohort of 97 patients with mCRC

	Univariate OS analysis				Multivariate OS Cox analysis			
		95% CI				95% CI		
	HR	Lower	Upper	Significance	HR	Lower	Upper	Significance
Age				0.363				–
< 70	1.00							
≥ 70	1.29	0.74 to 2.25			–	–		
Gender				0.227				–
Male	1.00							
Female	0.69	0.38 to 1.25			–	–		
Synchronous				0.096				–
No	1.00							
Yes	1.66	0.91 to 3.02			–	–		
ECOG				**< 0.001**				**< 0.001**
0–2	1.00				1.00			
3–4	2.04	1.46 to 2.84			1.86	1.32 to 2.62		
Number of metastatic sites				0.589				–
1–2	1.00							
> 2	1.13	0.71 to 1.80			-	–		
MiR-199b downregulation				**< 0.001**				**0.003**
No	1.00				1.00			
Yes	3.46	1.88 to 6.38			2.72	1.41 to 5.24		

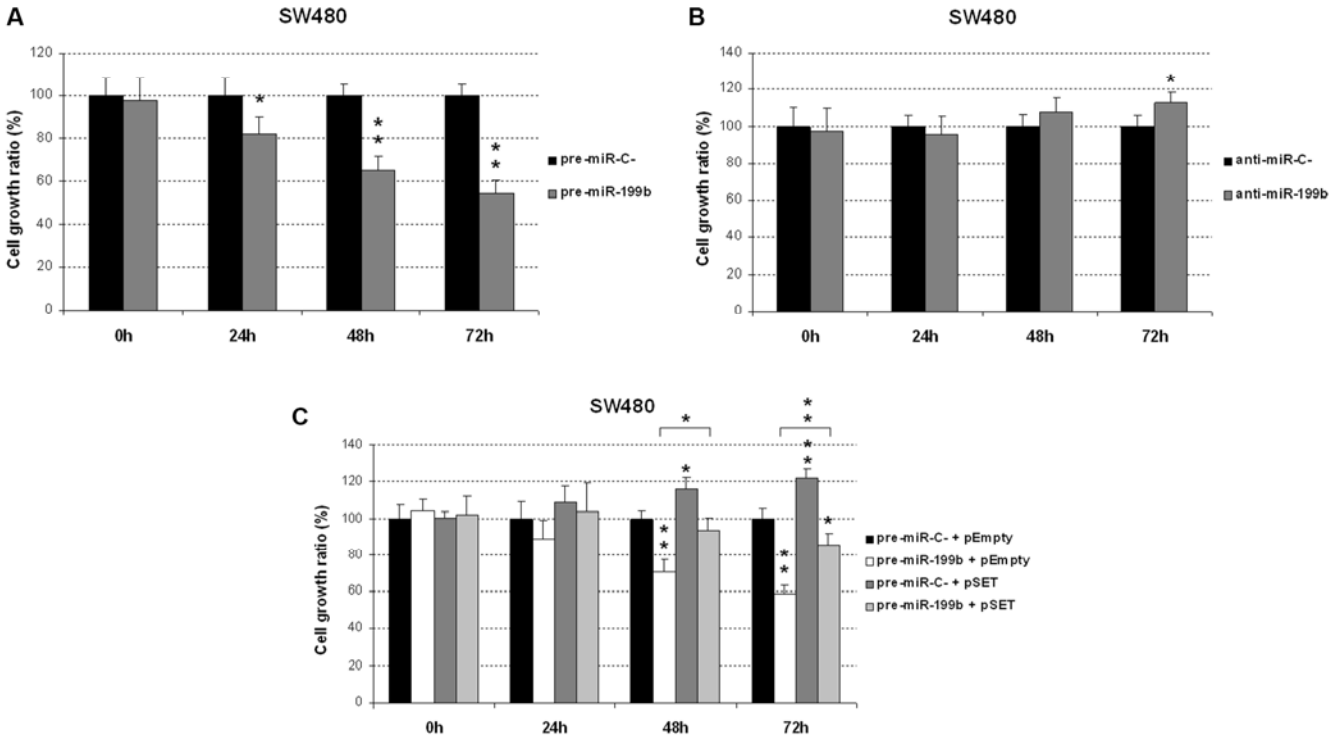


Figure 2: MiR-199b impairs cell proliferation in CRC cells. MTS assay showing proliferation in SW480 cells transfected with pre-miR-199b (**A**), anti-miR-199b (**B**) or both SET and pre-miR-199b (C). Data represented are mean of three independent experiments ± SD. **P* < 0.05; ***P* < 0.01.

Clinical significance of miR-199b downregulation in metastatic colorectal cancer

We next investigated the potential clinical significance of miR-199b in mCRC. Clinical follow-up data were available for all the 97 patients included in the study, 67 male and 30 female, with a median of age of 70 years (range: 40–89). The median OS of the global cohort was 25.3 months (95% confidence interval (CI): 16.2–34.4). Of relevance, we found that those patients with low miR-199b expression showed a substantially shorter OS (median OS, 9.7 versus 30 months, $p < 0.001$) (Figure 4A) and PFS (median PFS, 8.6 versus 15.4 months, $p = 0.003$) (Figure 4B).

We next stratified our cohort by *KRAS* mutation status, observing that miR-199b shows higher prognostic value in those patients with wild-type *KRAS* (median OS, 8.6 versus 30 months, $p = 0.001$; median PFS, 5.8 versus 15.4 months, $p = 0.017$) than in those cases with mutated *KRAS* (median OS, 13.5 versus 31.5 months, $p = 0.032$; median PFS, 8.7 versus 12.6 months, $p = 0.080$) (Figure 5). Moreover, miR-199b had significant prognostic value in OS in both subgroups of patients younger (median OS, 11.9 versus 34.2 months, $p = 0.003$) and older than 70 years (median OS, 3.9 versus 26.9 months, $p = 0.002$). Although miR-199b predicted PFS in younger patients (median PFS, 9.7 versus 22.5 months, $p = 0.009$), significance in PFS was not achieved in the subgroup of elderly cases (median PFS, 3.8 versus 12 months, $p = 0.119$) (Supplementary Figure S6). Importantly, we observed that miR-199b downregulation was predictive of clinical benefit in those patients who received oxaliplatin-based chemotherapy ($N = 39$; $p = 0.018$) (Table S3). Of relevance, multivariate analysis demonstrated that ECOG and miR-199b downregulation have an independent prognostic value in our patient cohort in both OS (Table 2) and PFS (Supplementary Table S4). Additionally, we also analyzed the clinical significance of the PP2A inhibitor SET in our series. Immunohistochemical detection of SET is shown in Supplementary Figure S4. As expected, we confirmed that those patients with SET overexpressed showed a substantially shorter OS (median OS, 9.9 versus 31.5 months, $p < 0.001$) and PFS (median PFS, 8.7 versus 18.5 months, $p = 0.009$) (Supplementary Figure S7).

Furthermore, we analyzed miR-199b levels in primary and paired liver metastatic tissues from 10 CRC patients in order to investigate the potential significance of miR-199b in CRC cell metastasis development. We quantified miR-199b levels using Taqman Low Density Arrays (TLDAs) panel A (Applied Biosystems). Interestingly, we found lower miR-199b levels in liver metastatic tissues compared to their paired primary CRC tissues ($p = 0.047$) (Supplementary Figure S8). Altogether, these preliminary results suggest that miR-199b overexpression could be playing a role in CRC liver metastasis development. Finally, we analyzed the potential role of miR-199b deregulation on *CD133* expression. We generated colonosphere-derived cells from the DLD-1, SW480 and HT-29 cell lines in which we observed *CD133* enrichment together with miR-199b downregulation (Supplementary Figure S9A). Although 3-fold increase in *CD133* expression was observed in DLD-1 colonospheres no expression of miR-199b was detected, similarly than in DLD-1 parental cells (data not shown). In addition, CD133

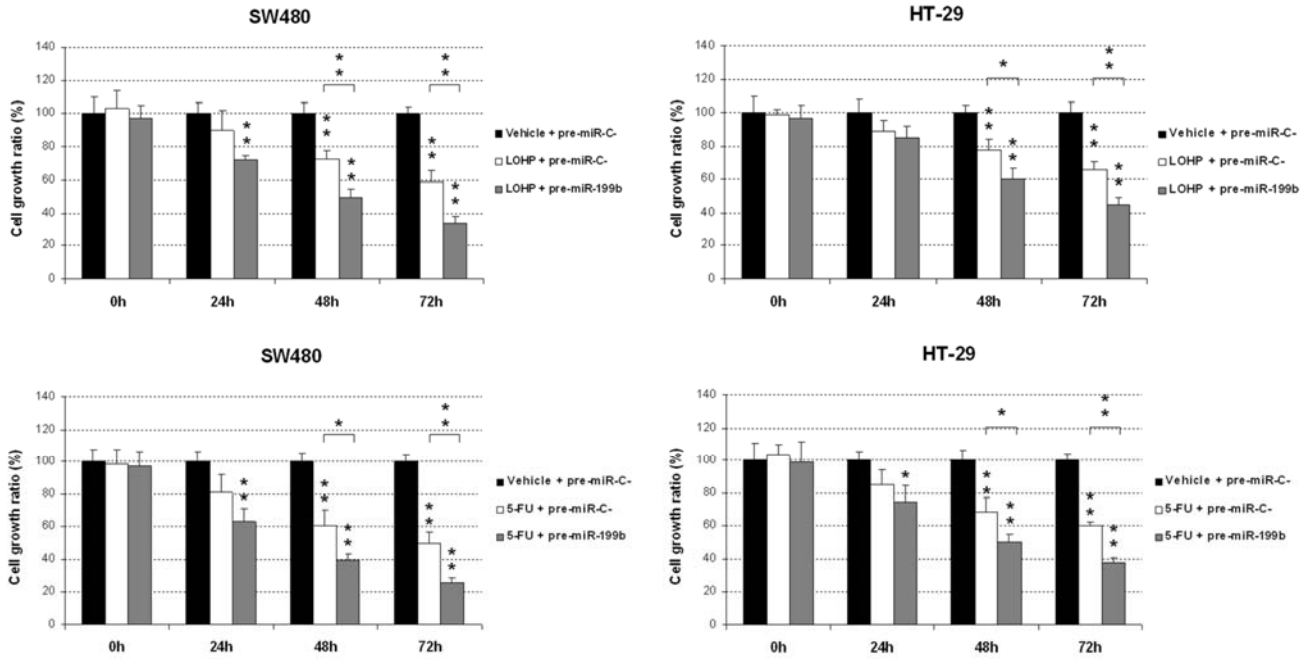


Figure 3: MiR-199b sensitizes CRC cells to oxaliplatin and 5-FU treatments. MTS assays showing the effect of miR-199b in the sensitivity to oxaliplatin (**A**) or 5-FU (**B**) in SW480 and HT-29 cells; Data represented are mean of three independent experiments ± SD. *$P < 0.05$; **$P < 0.01$.

expression could be quantified in 64 CRC patients from our cohort with enough material available. Interestingly, we found that miR-199b and *CD133* expression show a negative correlation (Supplementary Figure S9B), further suggesting that miR-199b deregulation could be affecting the *CD133* expression status of CRC cells.

DISCUSSION

Our group has recently reported that SET overexpression is a marker of poor outcome in metastatic CRC patients which defines a subgroup of patients candidate to be treated with PP2A activating drugs [18]. However, how SET deregulation occurs in CRC remains fully unknown. We then evaluated molecular causes that could lead to SET overexpression in CRC, analyzing whether an altered expression of miR-199b could be involved in SET deregulation in CRC. Thus, we first quantified miR-199b in 5 CRC cell lines previously reported to have SET overexpression [6], observing that miR-199b was downregulated in 4 out of 5 cases. This observation together with the significant lower miR-199b expression found in the subgroup of SET-overexpressing mCRC patients suggest a role of miR-199b in SET-mediated PP2A inhibition in CRC. This issue was further supported by the decrease in SET levels together with PP2A activation observed after miR-199b overexpression in CRC cell lines. Of note, the transfection with anti-miR-199b only promoted a slight reduction in PP2A activity probably due to the low basal miR-199b expression together with the SET overexpression status in both SW480 and HT-29 cells. These findings are in concordance with the fact that anti-miR-199b only induced a discrete increase of cell viability whereas miR-199b overexpression led to a marked reduction of cell growth. Moreover, the antitumor effects of miR-199b on cell growth is probably due to its role as negative SET regulator since the co-expression of miR-199b together with SET almost totally restored proliferation of CRC cells. Thus, miR-199b emerges as a novel tumor suppressor in CRC and its downregulation is a common alteration which contributes to PP2A inhibition in this disease.

Furthermore, miR-199b has been reported to be involved in acquired resistance to different antitumor therapies in human cancer such as imatinib in chronic myeloid leukemia [21], cisplatin in ovarian cancer [22] or trastuzumab in breast cancer [25]. Thus, we evaluated whether miR-199b could affect we observed that miR-199 sensitize CRC cells to both oxaliplatin and 5-FU treatments. These findings are concordant with the fact that miR-199b negatively regulates SET, which has been described to modulate resistance to oxaliplatin and 5-FU treatments in CRC [18].

Despite some data in the literature describe miR-199b tumor suppressor roles in human cancer [20–25], nothing is known about its function in CRC. As indicated above, miR-199b has been reported to have prognostic value in hepatocellular and papillary thyroid carcinomas [24, 26]. Therefore and considering that miR-199b seems to be a molecular cause of SET overexpression in a subgroup of metastatic CRC patients, we hypothesized that miR-199b downregulation could have clinical impact in metastatic CRC. Of importance, miR-199b downregulation determined poor outcome and clinical benefit in those cases treated with oxaliplatin-based chemotherapy. This observation is in concordance with our *in vitro* results and further supports that miR-199b increases sensitivity to oxaliplatin in CRC cells.

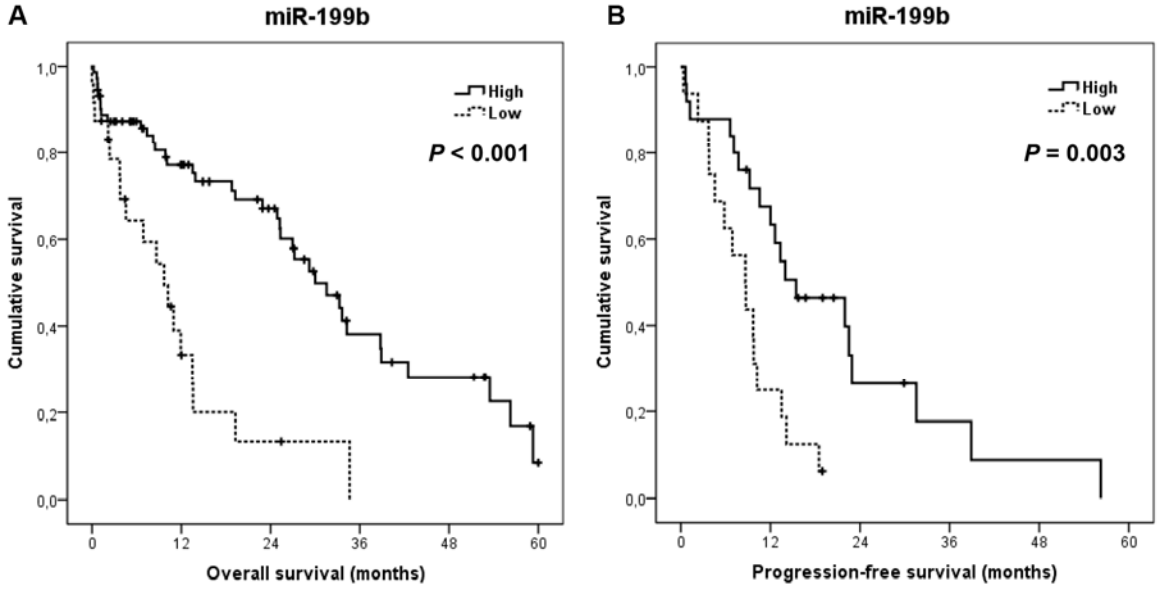


Figure 4: Clinical significance of miR-199b expression levels in metastatic CRC. Kaplan-Meier analyses of overall survival (**A**) and progression-free survival (**B**) in a cohort of 97 metastatic CRC patients.

The *KRAS* mutation status is a key molecular factor in determining clinical benefit to cetuximab in CRC [27]. Therefore, we evaluated the clinical impact of miR-199b stratifying our cohort by the presence or not of *KRAS* mutations. Thus, we found that miR-199b downregulation showed higher prognostic impact in both OS and PFS in the KRAS wild type subgroup. These findings could have a potential therapeutic relevance since FTY720, a PP2A-activating drug that binds and blocks SET [28], has recently shown to resensitize CRC cell to cetuximab [29] and our results suggest that miR-199b could be playing a role via SET regulation.

In addition to SET, miR-199b has been reported to regulate other important targets such as HEIS1, HIF1α or HER2 in medulloblastoma, hepatocellular carcinoma and breast cancers [23–25]. Among those metastatic CRC patients without SET overexpression, we observed miR-199b downregulated in 7 out of 65 cases. Of importance, we observed that miR-199b downregulation determined substantially shorter OS in these patients (median OS, 11 versus 31.5 months, $p = 0.052$), although significance was not achieved probably by the low number of cases studied. Therefore, these observations would indicate a potential SET-independent prognostic value for miR-199b which needs to be further confirmed in forthcoming studies. Moreover, multivariate analyses demonstrated that miR-199b downregulation was an unfavorable independent factor associated with OS and PFS in mCRC, which further confirm its prognostic value in this disease.

In concordance with previous observations in medulloblastoma [23], we show lower miR-199b

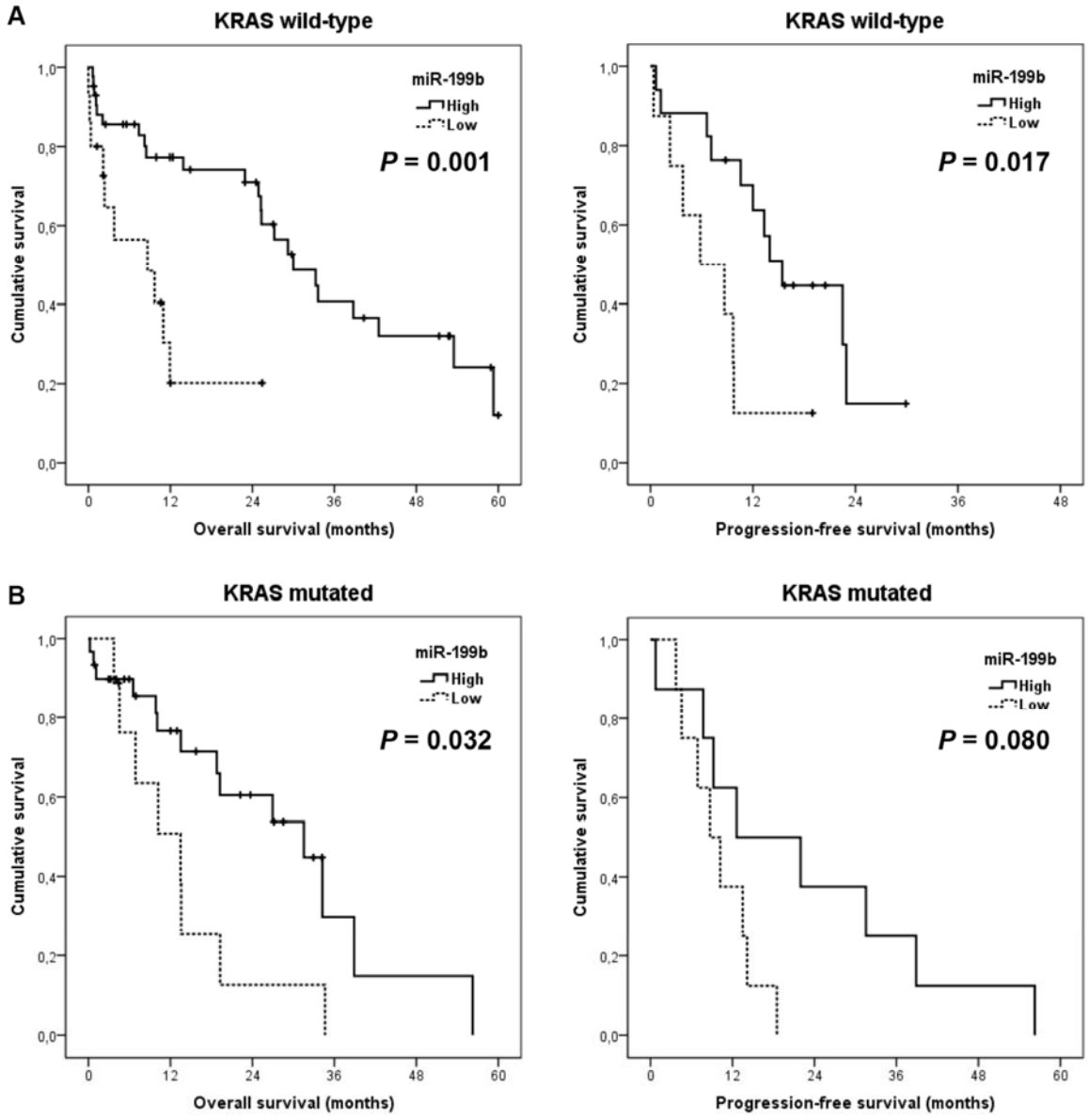


Figure 5: Prognostic impact of miR-199b in metastatic CRC patients stratified by *KRAS* mutation status. Kaplan-Meier analyses of overall survival and progression-free survival in *KRAS* wild type ($N = 58$) (**A**) and *KRAS* mutated subgroups ($N = 39$) (**B**).

expression in CRC with metastatic disease. In their work, Garzia et al. provided relevant findings supporting that miR-199b downregulation in metastatic medulloblastoma cells was probably due to a methylation-based epigenetic regulation of this microRNA. Therefore, it remains necessary to evaluate in future investigations whether a similar mechanism of transcriptional regulation is also occurring in CRC cells. On the other hand, the Notch signaling pathway plays a relevant role in self-renewing processes and its inhibition has been described to decrease CD133+ tumor cells [23, 30]. Of importance, a negative feedback loop of regulation has been reported between miR-199b and HES1, a key Notch effector, then impairing the CD133+ stem cell-like subpopulation of tumor cells [23, 31]. Interestingly, we show here that miR-199b is downregulated after colonosphere generation, which are CD133-enriched cells. Moreover, we analyzed *CD133* in 64 metastatic CRC patients observing a negative correlation between *CD133* and miR-199b. These results would indicate a potential relationship between miR-199b and CD133 in CRC cells that needs to be further explored in forthcoming studies.

In conclusion, our results show that miR-199 downregulation is a frequent alteration in metastatic CRC that emerges as a novel therapeutic target and a contributing mechanism to SET overexpression in this disease. Interestingly, our findings indicate that miR-199 downregulation is a common event that plays an oncogenic role in CRC cells. Moreover, this alteration has an independent prognostic value predicting poor outcome in metastatic CRC patients and could have important therapeutic implications via SET-dependent PP2A inhibition in metastatic CRC.

MATERIALS AND METHODS

Cell cultures and transfection

The human CRC cell lines SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38) and SW620 (ATCC CCL-227) were purchased from American Type Culture Collection (ATCC). Authentication was done by the authors in all cases (LGC Standards). Cell lines were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum and were grown at 37ºC in a 5% CO2 atmosphere. Media were supplemented with penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). Cells were treated with oxaliplatin (LOHP) (1 μM) (Sigma), 5-fluorouracil (5-FU) (1 μM) (Sigma) and FTY720 (10 μM) (Calbiochem) as previously reported [6, 9]. For transfection experiments, CRC cells were seeded in 6-well plates and transfected with 10 μl of Lipofectamine 2000 (Life Technologies) and 2 μg of SET plasmidic vector or 20 nM of a miR-199b specific *mir*Vana™ miRNA Mimic and Inhibitor (Ambion).

Patient samples

Primary colorectal tissues were surgical resection specimens from CRC tumors obtained from Fundacion Jimenez Diaz Biobank (BFJD, Madrid). The study comprised consecutive FFPE tumor samples of 97 patients with metastatic CRC that were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data and metastatic disease. TNM (Tumor, Node, Metastases) staging was classified using the 7th American Joint Committee on Cancer (AJCC) staging system for colorectal cancer. Clinical data were collected from medical clinical records by oncologists. KRAS mutational status was determined by Cobas KRAS Mutation Test kit (Roche Molecular Diagnostics) following manufacturer's procedures. Tissue microarrays (TMA) were constructed. Representative areas of each tumor were carefully selected and three tissue cores (1 mm diameter) were obtained using a TMA workstation (T1000 Chemicon). Samples were taken anonymously. The ethical committee and institutional review board approved the project.

Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following manufacturer´s indications, clarified (12,000 × g, 15 min, 4ºC), denatured and subjected to SDS-PAGE and Western-blot. Antibodies used were rabbit polyclonal anti-SET (Abcam) and mouse monoclonal anti-βactin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phospatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems).

Cell viability assay

Cell proliferation was measured in triplicate wells by MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer´s indications.

PP2A phosphatase activity assay

PP2A assays were performed with cell lysates (50 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described [6].

Immunohistochemistry

Tissue sections (3 μm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 min. Slides

were incubated with same primary antibody against SET as described for 60 minutes at room temperature, followed of appropriate anti-Ig horseradish peroxidase-conjugated polymer (Flex+, Dako). Sections were visualized with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with normal non-immunized rabbit immunoglobulins were used as negative controls. As positive control, a section of colorectal tumor with known expression of SET was used. SET antibody sensitivity (1:5000) had been calculated in a range of crescent dilutions of primary antibody. Specificity was confirmed in a set of paired fresh frozen and FFPE samples were processed by western blot and IHC. Only the membrane of epithelial cells, but not stromal cells, was evaluated for SET expression blinded to clinical data by two pathologists. A semiquantitative histoscore was calculated by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low %) × 1 + (medium %) × 2 + (high %) × 3 and the results ranged from 0 to 300.

Quantification of miRNA expression levels

Total RNA was isolated using RecoverAll Total Nucleic Acid Isolation kit (Ambion) according to manufacturer's instructions. Samples were reverse transcribed using the TaqManHMicroRNA Reverse Transcription Kit (Applied Biosystems) and mature miRNAs were quantified by quantitative real-time RT-PCR using TaqMan MicroRNA Assays (Applied Biosystems) specific for miR-199b (miR-199b-5p_000500) and U6B as internal control. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C}$T method [32]. The mean expression value of the global cohort (ΔCtcohort) was used to obtain the relative expression of each sample (ΔΔCt) and the fold change calculated as $2^{-\Delta\Delta C}$T. Downregulation of miR-199b was considered when the expression in a sample was lower than mean minus SD of the patient cohort, corresponding to 0.378 fold change.

Luciferase assays

Luciferase assays were done using the Dual-Glo Luciferase Assay System (Promega) following the manufacturer´s intructions. SW480 cells were transfected with 20 nM of prc-miR-199b (Ambion) and a pmiR-Glo construct empty or including the SET 3´UTR with the wild type or mutated miR-199b seed region. Firefly luciferase activities were normalized to Renilla luciferase activities.

Colonospheres

We generated colonosphere-derived cells from DLD-1, SW480 and HT-29 cells using 6-well ultra-low attachment plates (Corning) and 10,000 cells per well. Cells were grown in serum-free DMEM/F12 supplemented with GlutMAX™-I (Gibco) 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/ml human FGF (Sigma) and 50 ng/ml EGF (Sigma). After 7 days, plates were analyzed for colonosphere formation.

Statistical analysis

Statistical analyses were performed using SPSS 20 for windows (SPSS Inc, Chicago Illinois). Overall survival (OS) was defined as the time from the date of surgery to the date of death from any cause or last follow-up. Progression-free survival (PFS) was defined as the time from surgery until any primary, regional or distant recurrence, appearance of a secondary tumor or death. Kaplan-Meier method and survival comparisons were done with the log-rank test if proportional hazard assumption was fulfilled and Breslow otherwise. The Cox proportional hazards model was adjusted taking into consideration significant parameters in univariate analysis. A *P*-value less than 0.05 was considered statistically significant. Receiver operating curve (ROC) was used to determine the optimal cutoff point based on progression end point for SET expression as previously described method to calculate threshold values for biomarker analysis [33–35]. Following this criteria, high SET exprssion was considered when Hscore in tumor cells were equal or higher than 100. This work was carried out in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [36].

ACKNOWLEDGMENTS

None.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FINANCIAL SUPPORT

This work was supported by PI13/02609 and PI15/00934 grants from Instituto de Salud Carlos III. BT and PG-A are supported by Fundación Conchita Rábago de Jiménez Díaz.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69–90.
2. Lombardi L, Morelli F, Cinieri S, Santini D, Silvestris N, Fazio N, Orlando L, Tonini G, Colucci G, Maiello E. Adjuvant colon cancer chemotherapy: where we are and where we'll go. Cancer Treat Rev. 2010; 36:S34–41.

3. Gill S, Blackstock AW, Goldberg RM. Colorectal cancer. Mayo Clin Proc. 2007; 82:114–29.
4. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell. 2007; 130:21–4.
5. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol. Med. 2008; 14:152–60.
6. Cristobal I, Manso R, Rincon R, Carames C, Senin C, Borrero A, Martínez-Useros J, Rodriguez M, Zazo S, Aguilera O, Madoz-Gúrpide J, Rojo F, García-Foncillas J. PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. Mol Cancer Ther. 2014;13:938–47.
7. Carmen Figueroa-Aldariz M, Castañeda-Patlán MC, Santoyo-Ramos P, Zentella A, Robles-Flores M. Protein phosphatase 2A is essential to maintain active Wnt signaling and its Aβ tumor suppressor subunit is not expressed in colon cancer cells. Mol Carcinog. 2014; 54:1430–41.
8. Cristóbal I, Manso R, Rincón R, Caramés C, Zazo S, Del Pulgar TG, Madoz-Gúrpide J, Rojo F, García-Foncillas J. Phosphorylated protein phosphatase 2A determines poor outcome in patients with metastatic colorectal cancer. Br J Cancer. 2014; 111:756–62.
9. Cristóbal I, Rincón R, Manso R, Madoz-Gúrpide J, Caramés C, del Puerto-Nevado L, Rojo F, García-Foncillas J. Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation. Biochim Biophys Acta. 2014; 1842:1823–9.
10. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J Biol Chem. 1996; 271:11059–62.
11. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell. 2001; 104:119–30.
12. ten Klooster JP, Leeuwen I, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET. Embo J. 2007; 26:336–45.
13. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, Diaz C, Pujol MJ, Agell N, Bachs O. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. J Biol Chem. 2003; 278:1158–64.
14. Trotta R, Ciarlariello D, Dal Col J, Allard J 2nd, Neviani P, Santhanam R, Mao H, Becknell B, Yu J, Ferketich AK, Thomas B, Modi A, Blaser BW, et al. The PP2A inhibitor SET regulates natural killer cell IFN-gamma production. J Exp Med. 2007; 204:23397–405.
15. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem. 2002; 277:25026–31.
16. Switzer CH, Cheng RY, Vitek TM, Christensen DJ, Wink DA, Vitek MP. Targeting SET/I(2)PP2A oncoprotein functions as a multi-pathway strategy for cancer therapy. Oncogene. 2011; 30:2504–13.
17. Dong L, Zhu J, Wen X, Jiang T, Chen Y. Involvement of SET in the Wnt signaling pathway and the development of human colorectal cancer. Oncol Lett. 2014; 7:1203–8.
18. Cristóbal I, Rincón R, Manso R, Caramés C, Zazo S, Madoz-Gúrpide J, Rojo F, García-Foncillas J. Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer. Clin Cancer Res. 2015; 21:347–56.
19. Chao A, Tsai CL, Wei PC, Hsueh S, Chao AS, Wang CJ, Tsai CN, Lee YS, Wang TH, Lai CH. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. Cancer Lett. 2010; 291:99–107.
20. Cristóbal I, Garcia-Orti L, Cirauqui C, Cortes-Lavaud X, García-Sánchez MA, Calasanz MJ, Odero MD. Overexpression of SET is a recurrent event associated with poor outcome and contributes to protein phosphatase 2A inhibition in acute myeloid leukemia. Haematologica. 2012; 97:543–50.
21. Joshi D, Chandrakala S, Korgaonkar S, Ghosh K, Vundinti BR. Down-regulation of miR-199b associated with imatinib drug resistance in 9q34.1 deleted BCR/ABL positive CML patients. Gene. 2014; 542:109–12.
22. Liu MX, Siu MK, Liu SS, Yam JW, Ngan HY, Chan DW. Epigenetic silencing of microRNA-199b-5p is associated with acquired chemoresistance via activation of JAG1-Notch1 signaling in ovarian cancer. Oncotarget. 2014; 5:944–58. doi: 10.18632/oncotarget.1458.
23. Garzia L, Andolfo I, Cusanelli E, Marino N, Petrosino G, De Martino D, Esposito V, Galeone A, Navas L, Esposito S, Gargiulo S, Fattet S, Donofrio V, et al. MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. PLoS One. 2009; 4:e4998.
24. Wang C, Song B, Song W, Liu J, Sun A, Wu D, Yu H, Lian J, Chen L, Han J. Underexpressed microRNA-199b-5p targets hypoxia-inducible factor-1α in hepatocellular carcinoma and predicts prognosis of hepatocellular carcinoma patients. J Gastroenterol Hepatol. 2011; 26:1630–7.
25. Fang C, Zhao Y, Guo B. MiR-199b-5p targets HER2 in breast cancer cells. J Cell Biochem. 2013; 114:1457–63.
26. Peng Y, Li C, Luo DC, Ding JW, Zhang W, Pan G. Expression profile and clinical significance of microRNAs in papillary thyroid carcinoma. Molecules. 2014; 19:11586–99.
27. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.

28. Saddoughi SA, Gencer S, Peterson YK, Ward KE, Mukhopadhyay A, Oaks J, Bielawski J, Szulc ZM, Thomas RJ, Selvam SP, Senkal CE, Garrett-Mayer E, De Palma RM, et al. Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumor suppression via activation of PP2A-RIPK1-dependent necroptosis. EMBO Mol Med. 2013; 5:105–21.

29. Rosa R, Marciano R, Malapelle U, Formisano L, Nappi L, D´Amato C, D'Amato V, Damiano V, Marfè G, Del Vecchio S, Zannetti A, Greco A, De Stefano A, et al. Sphingosine kinase 1 overexpression contributes to cetuximab resistance in human colorectal cancer models. Clin Cancer Res. 2013; 19:138–47.

30. Fan X, Matsui W, Khaki L, Stearns D, Chun J, Li YM, Eberhart CG. Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. Cancer Res. 2006; 66:7445–52.

31. Andolfo I, Liguori L, De Antonellis P, Cusanelli E, Marinaro F, Pistollato F, Garzia L, De Vita G, Petrosino G, Accordi B, Migliorati R, Basso G, Iolascon A, et al. The micro-RNA 199b-5p regulatory circuit involves Hes1, CD15, and epigenetic modifications in medulloblastoma. Neuro Oncol. 2012; 14:596–612.

32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–8.

33. Obuchowski NA. ROC analysis. AJR Am J Roentgenol. 2005; 184,364–72.

34. Generali D, Buffa FM, Berruti A, Brizzi MP, Campo L, Bonardi S, Bersiga A, Allevi G, Milani M, Aguggini S, Papotti M, Dogliotti L, Bottini A, et al Phosphorylated ERalpha, HIF-1alpha, and MAPK signaling as predictors of primary endocrine treatment response and resistance in patients with breast cancer. J Clin Oncol. 2009; 27:227–34.

35. Caramés C, Cristóbal I, Moreno V, del Puerto L, Moreno I, Rodriguez M, Marin JP, Correa AV, Hernandez R, Zenzola V, Hernandez T, Leon A, Martin JI, et al. MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. Int J Colorectal Dis. 2015; 30:899–906.

36. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clarck GM. Reporting recommendations for tumor marker prognostic studies. J Clin Oncol. 2005; 23:9067–72.

Downregulation of microRNA-199b predicts unfavorable prognosis and emerges as a novel therapeutic target which contributes to PP2A inhibition in metastatic colorectal cancer

Supplementary Materials

Supplementary Table S1: Quantification of the expression levels of hsa-mir-199b by real-time PCR in 5 CRC cell lines

Cell line	SET OE	Mir-199b ($-\Delta\Delta C_T$)
SW480	yes	−4,89
WiDr	yes	No expression
DLD-1	yes	No expression
HT-29	yes	−4,30
SW620	yes	−0,25

OE: Overexpression.
$\Delta\Delta C_T = (C_{T,miR\text{-}199b} - C_{T,U6B})_{Cell\ Line} - (C_{T,Target\ Gene} - _{CT,U6B})$Normal Controls. The mean mir-199b expression from a set of 8 normal colonic mucosa samples was used as normal control in this experiment.

Supplementary Table S2: Clinical and molecular characteristics of a series of 97 patients with metastatic CRC

		No. (%)
Sex		
Male	67	(69.1)
Female	30	(30.9)
Age		
< 70	44	(47.3)
≥ 70	49	(52.7)
No data	4	
ECOG		
0–2	75	(81.5)
3–4	17	(18.5)
No data	5	
MSI		
No	89	(93.7)
Yes	6	(6.3)
No data	2	
KRAS mutations		
No	58	(59.8)
Yes	39	(40.2)
Site of primary tumor		
Colon	72	(74.2)
Rectum	25	(25.8)
Synchronous metastasis		
No	39	(40.2)
Yes	58	(59.8)
Number of metastatic sites		
1–2	89	(91.8)
> 2	8	(8.2)
Liver metastasis		
No	33	(34)
Yes	64	(66)
Lung metastasis		
No	68	(70.1)
Yes	29	(29.9)
Lymph metastasis*		
No	68	(70.1)
Yes	29	(29.9)
Peritoneal metastasis		
No	78	(80.4)
Yes	19	(19.6)
Prior adjuvant chemotherapy**		
No	19	(48.7)
Yes	20	(51.3)
Treatment 1st line metastatic		
Oxaliplatin	39	(41.5)
Irinotecan	13	(13.8)
5-FU	9	(9.6)
None	33	(35.1)
No data	3	

*Non-regional lymph node involvement; **Cases with metachronous metastasis only.

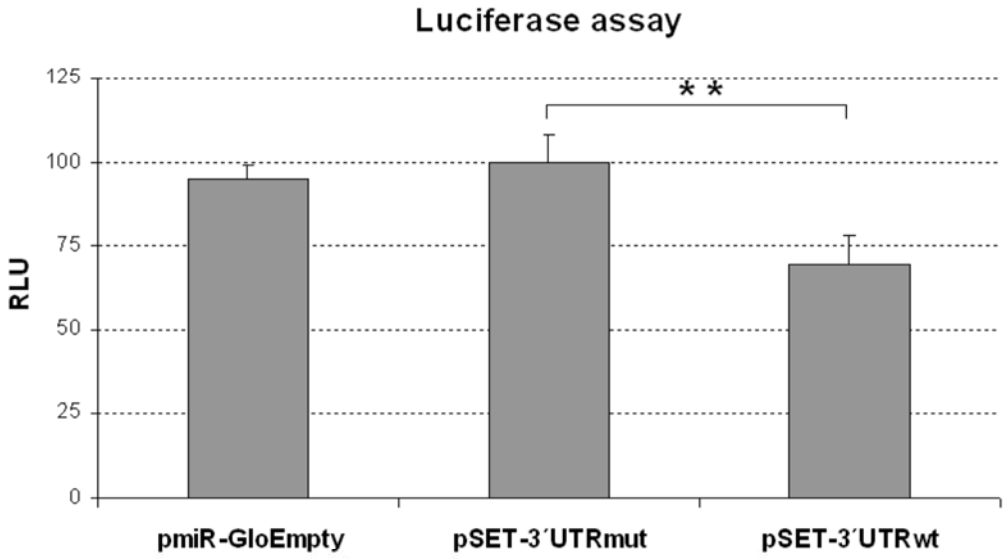
Supplementary Table S3: Predictive value of response to treatment by miR-199b deregulation in those patients who received oxaliplatin-based chemotherapy

	Response			
	Total	**No progression (%)**	**Progression (%)**	***p***
Low miR-199b	39	14	25	**0.018**
No	31	14 (100)	17 (68)	
Yes	8	0 (0)	8 (32)	

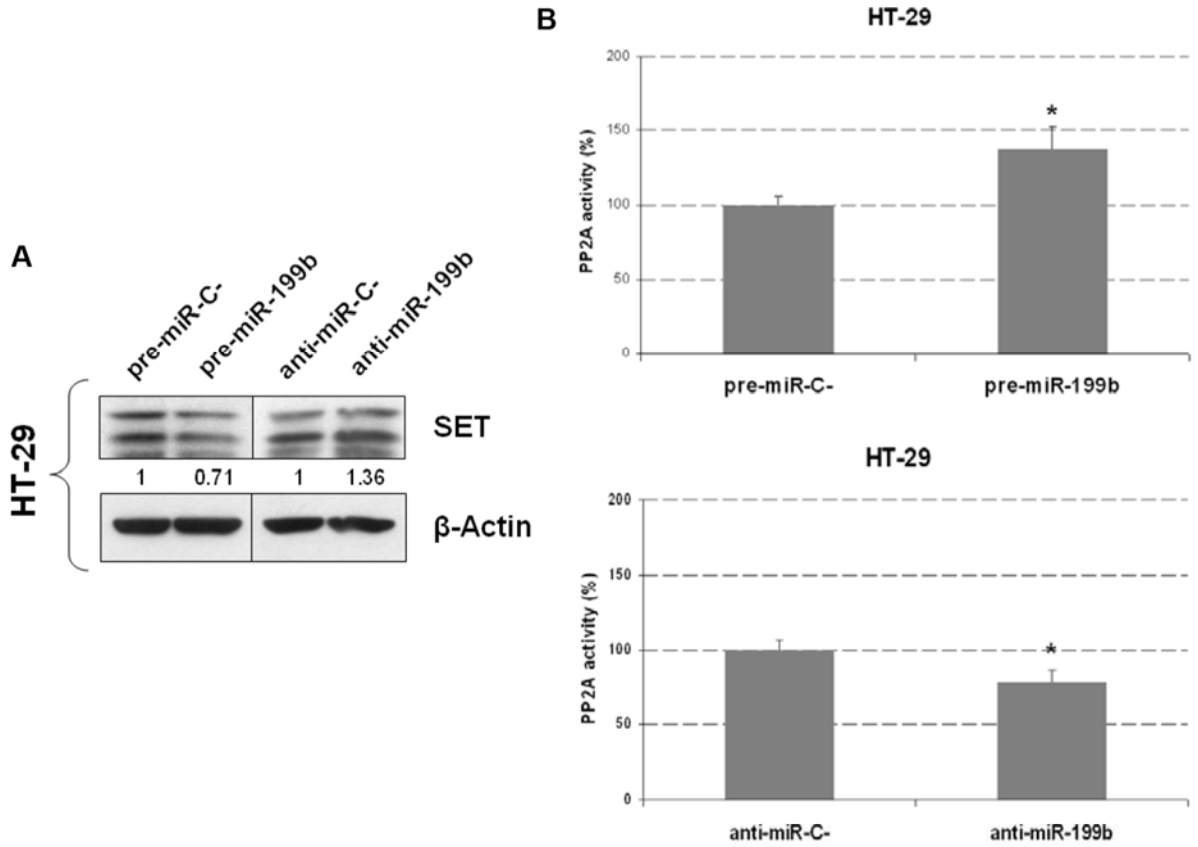
The Fisher exact test was applied to calculate the *p*-value.

Suppmentary Table S4: Univariate and multivariate Cox analyses in the cohort of 97 patients with metastatic CRC

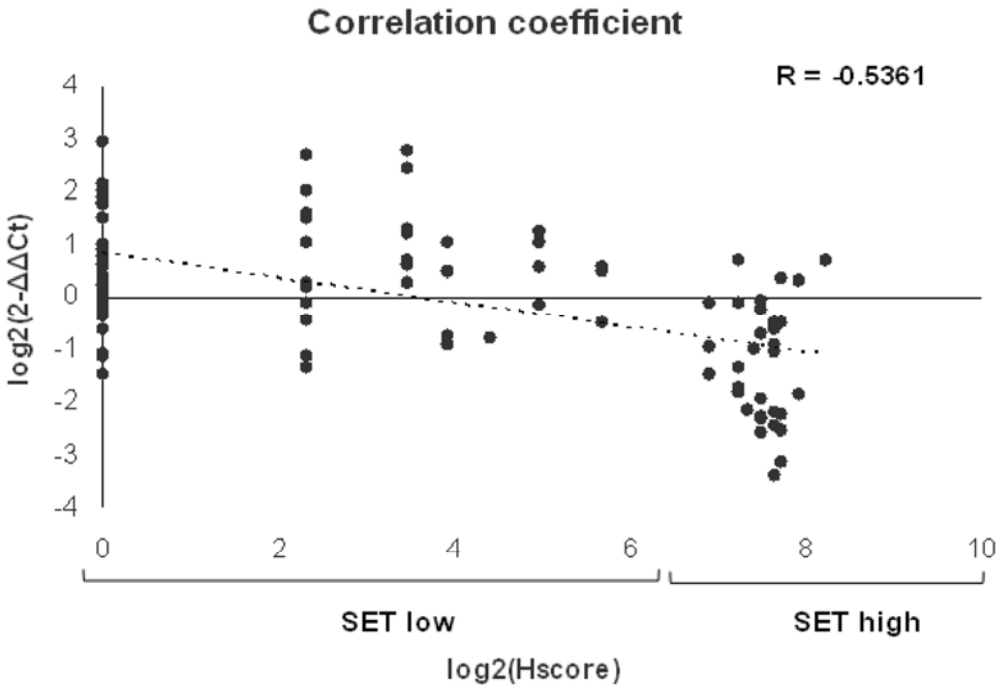
		Univariate PFS analysis				Multivariate PFS Cox analysis			
			95% CI				95% CI		
		HR	**Lower**	**Upper**	**Significance**	**HR**	**Lower**	**Upper**	**Significance**
Age					0.102				–
	< 70	1.00							
	≥ 70	1.79	0.89 to 3.62			–	–		
Gender					0.352				–
	Male	1.00							
	Female	0.71	0.34 to 1.45			–	–		
Synchronous					0.480				–
	No	1.00							
	Yes	1.32	0.61 to 2.86			–	–		
ECOG					**0.008**				**0.040**
	0–2	1.00				1.00			
	3–4	1.72	1.15 to 2.57			1.54	1.01 to 2.34		
Number of metastatic sites					0.225				–
	1–2	1.00							
	> 2	1.36	0.82 to 2.26			–	–		
MiR-199b downregulation					**0.005**				**0.037**
	No	1.00				1.00			
	Yes	2.94	1.38 to 6.27			2.32	1.05 to 5.13		



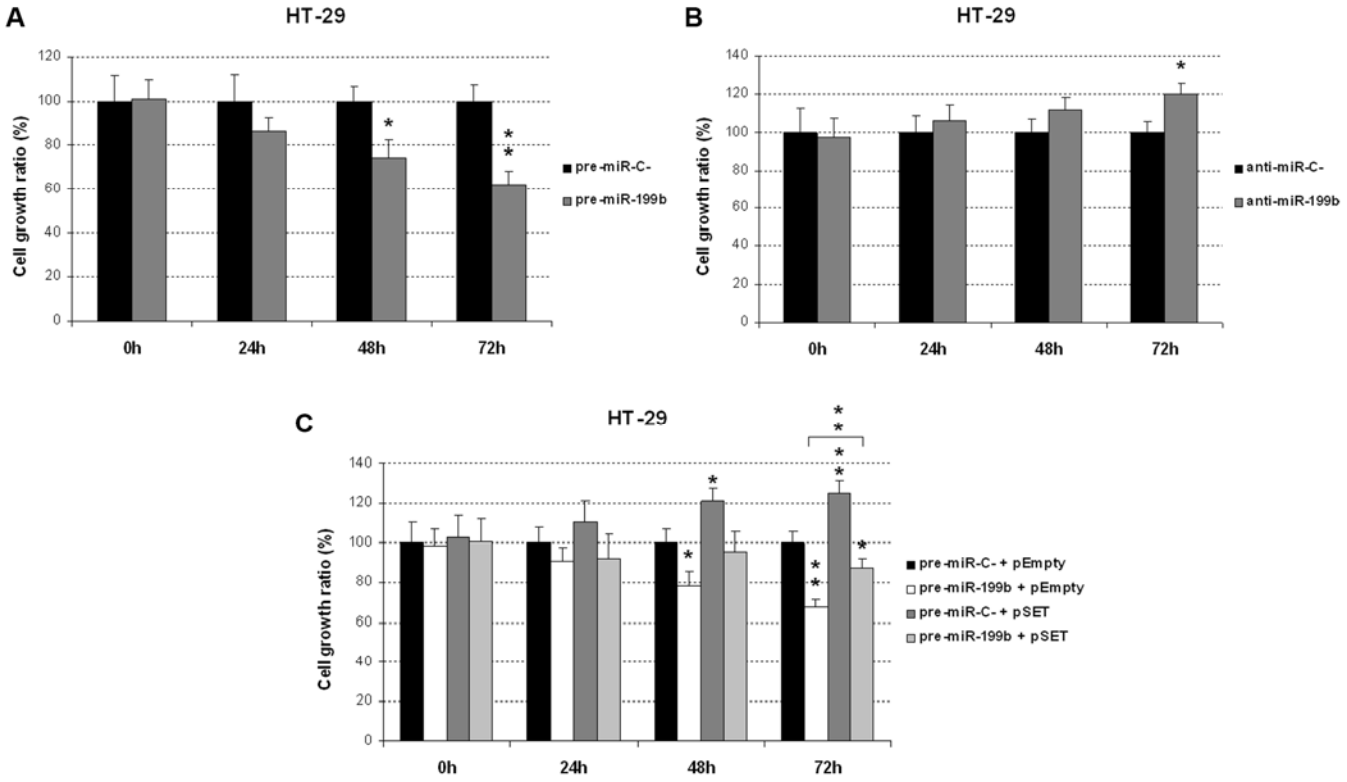
Supplementary Figure S1: Luciferase assay showing changes in Firefly luciferase activity in SW480 cells ectopically expressing miR-199b and transfected with pmiR-Glo empty (negative control), or a pmiR-Glo vector with the 3′UTR region of SET that includes the miR-199b seed region (pSET-3′UTRwt). Transfection with the 3′UTR region of SET including a mutated seed region for miR-199b (pSET-3′UTRmut) was used as reference control. Results were normalized to Renilla luciferase activity and represented as relative luminescence units (RLU). Data represented are mean of three independent experiments ± SD. $^{**}P < 0.01$.



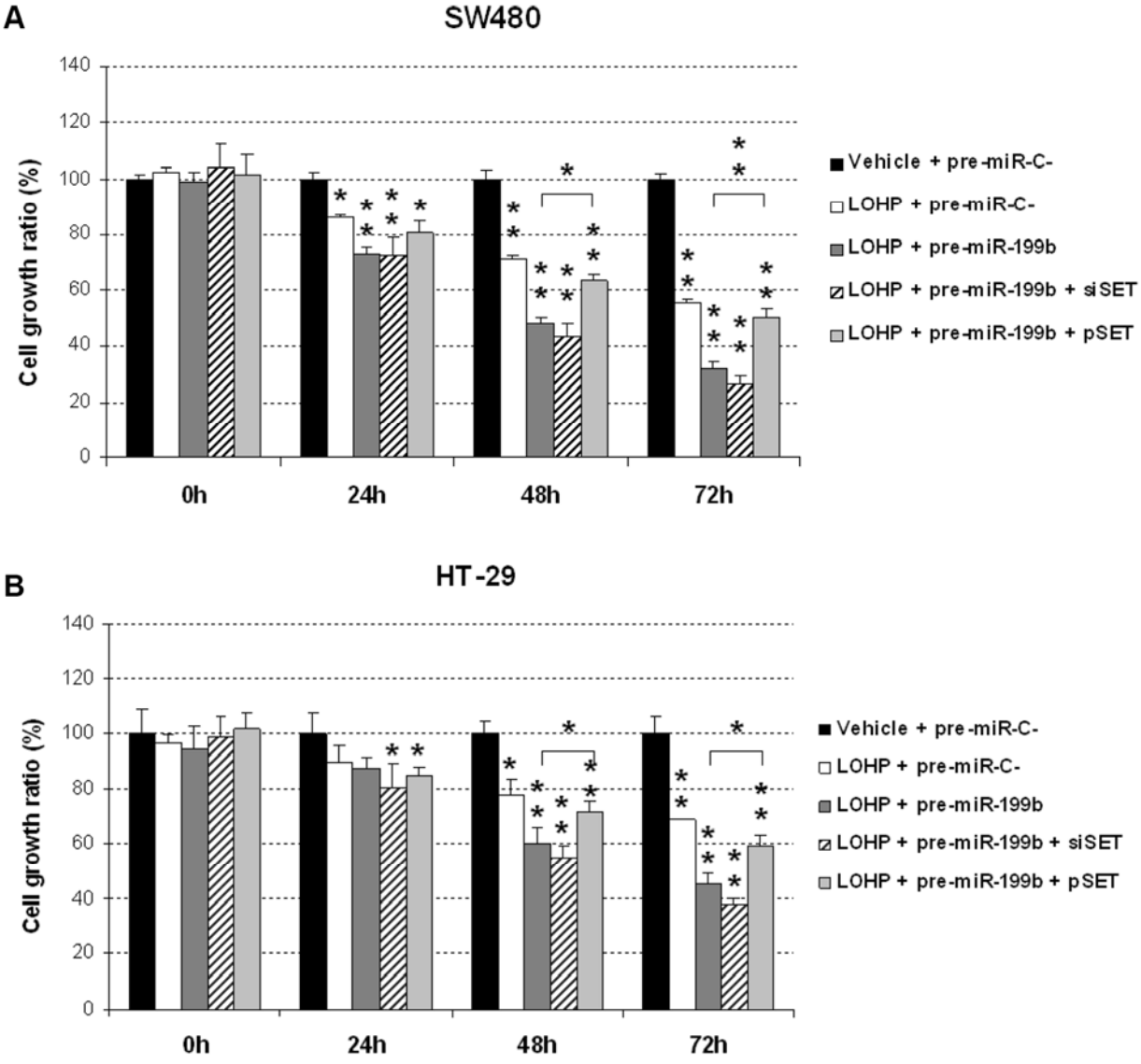
Supplementary Figure S2: (A) Western blot analysis showing SET expression in HT-29 cells transfected with pre- or anti-miR-199b; (B) PP2A assay showing changes in PP2A activity after transfection with pre- or anti-miR-199b.



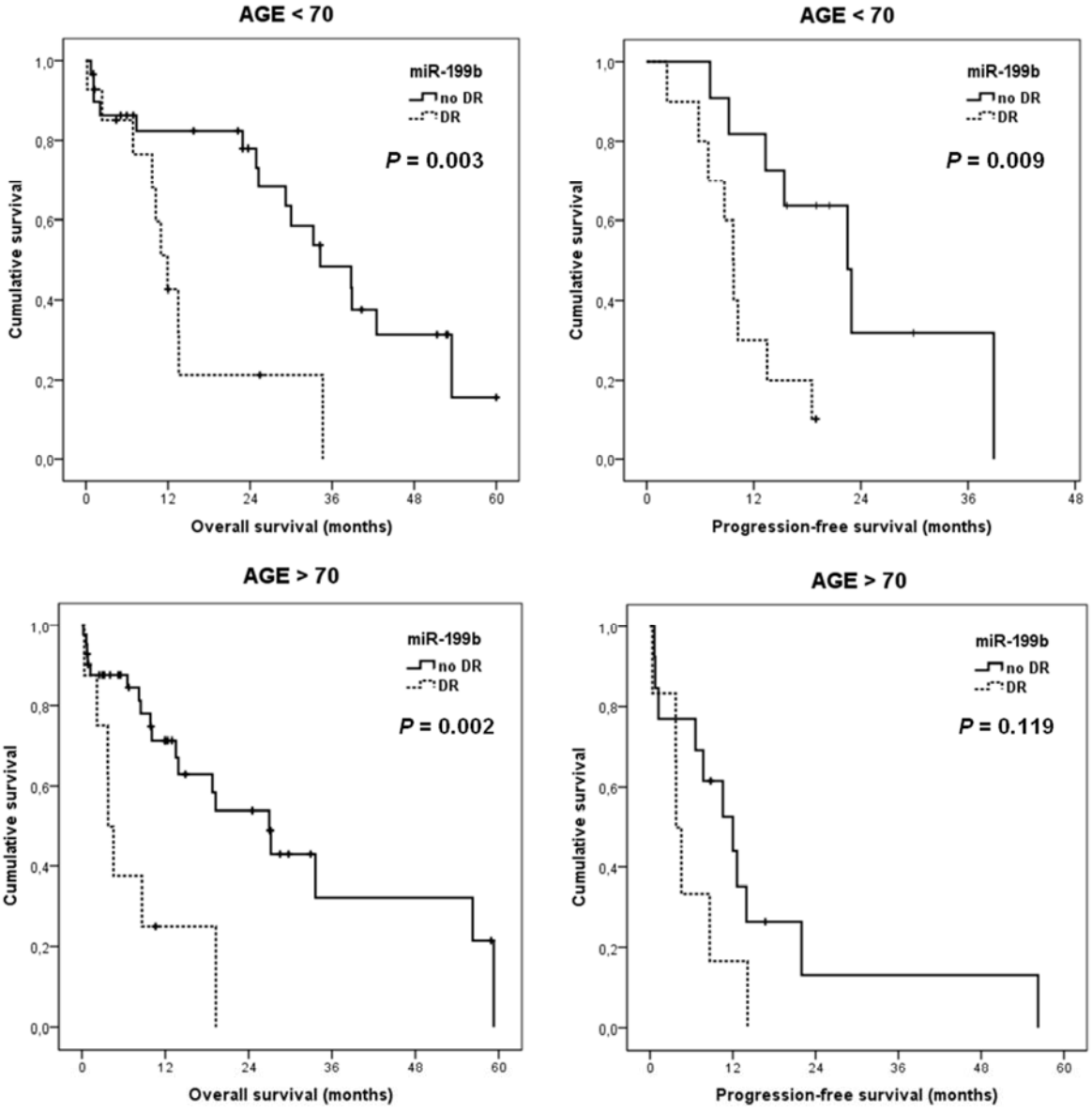
Supplementary Figure S3: Scatter plot showing the correlation between miR-199b and SET expression in 97 CRC patients.



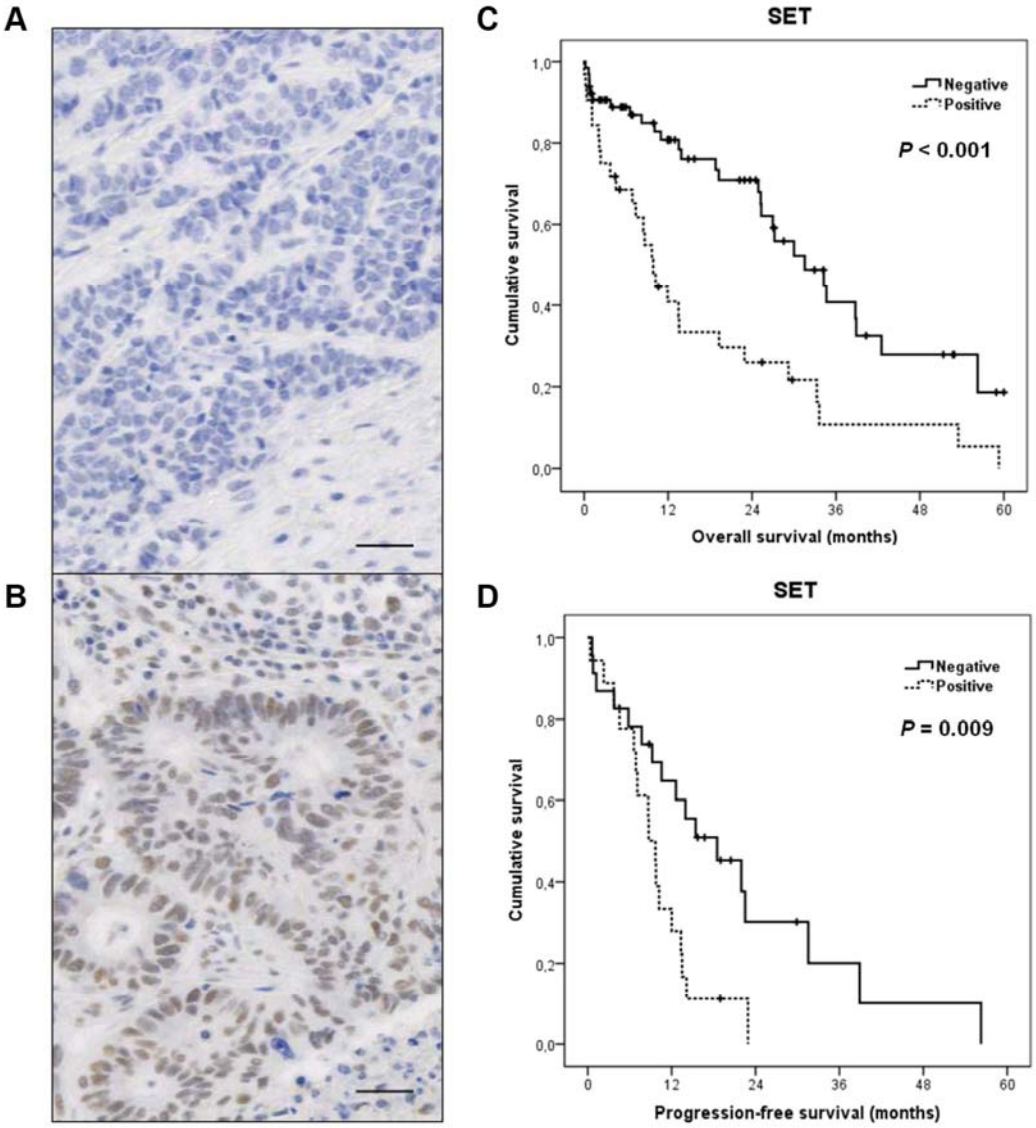
Supplementary Figure S4: MTS assay showing proliferation in HT-29 cells transfected with pre-miR-199b (A), anti-miR-199b (B) or both SET and pre-miR-199b (C); *$P < 0.05$; **$P < 0.01$.



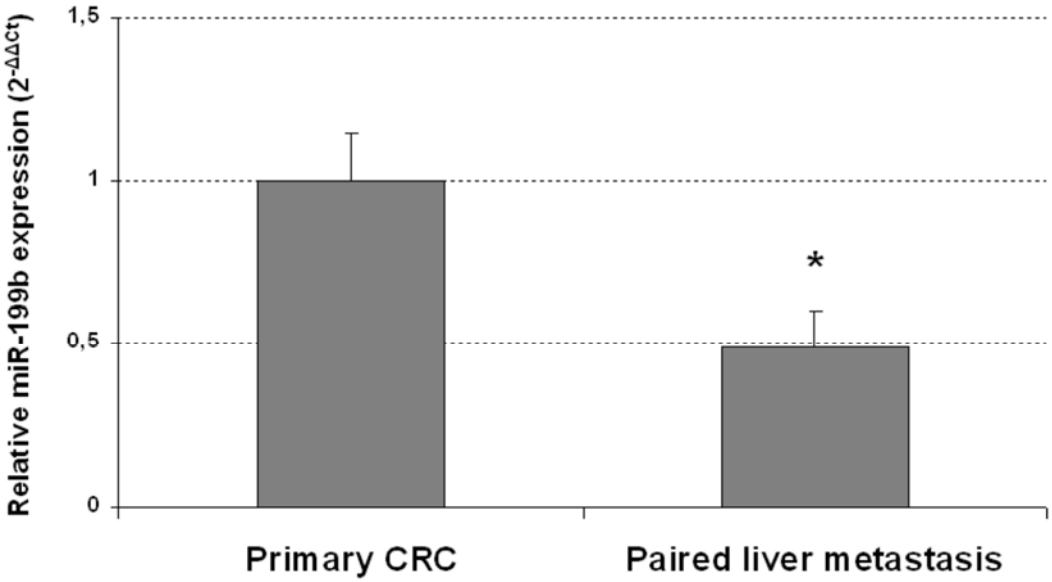
Supplementary Figure S5: MTS assay showing effects of SET modulation in miR-199b-dependent oxaliplain re-sensitation in SW480 (A) and HT-29 cells (B); *$P < 0.05$; **$P < 0.01$



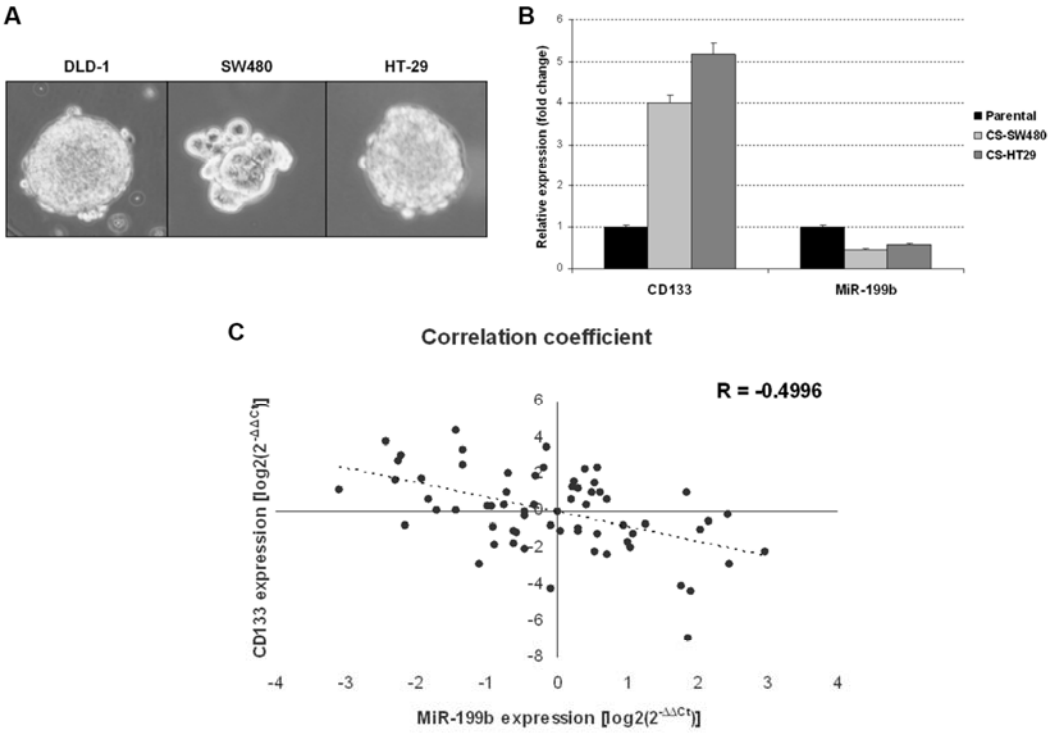
Supplementary Figure S6: Kaplan-Meier analyses of overall and progression-free survival and in the subgroups of patients younger (*N* = 44) and older than 70 years (*N* = 49).



Supplementary Figure S7: Immunohistochemical detection of SET expression in patients with metastatic colorectal cancer. SET negative (**A**) and positive (**B**) staining. The line in A and B shows 25 µm. Magnification x400. Kaplan-Meier analyses for SET in a cohort of 97 patients with metastatic CRC: (**C**) Overall survival; (**D**) Progression-free survival.



Supplementary Figure S8: Relative miR-199b expression in 10 CRC primary tumors compared with paired liver metastases. A related-samples Wilcoxon signed-ranked test was performed to assess statistical differences. *$P < 0.05$.



Supplementary Figure S9: (A) Optical microscope images showing DLD-1, SW480 and HT-29-derived colonospheres; (**B**) Quantification of miR-199b and CD133 expression in parental and colonosphere (CS)-derived SW480 and HT-29 cells. We used TaqMan Gene Expression Assays specific for *CD133* (Hs01009259_m1) and GAPDH as internal control; (**C**) Scatter plot showing the correlation between miR-199b and *CD133* expression in 64 metastatic CRC patients.

Article 6: MicroRNA-31 emerges as a predictive biomarker of pathological response and outcome in locally advanced rectal cancer.[6]

Int J Colorectal Dis
DOI 10.1007/s00384-015-2231-9

ORIGINAL ARTICLE

MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer

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Accepted: 28 April 2015

Abstract
Purpose The treatment of choice for locally advanced rectal cancer is preoperative chemoradiotherapy. Despite half of patients do not respond and suffer unnecessary toxicities and surgery delays, there are no biomarkers to guide preoperative CRT outcome. MicroRNA-21 has been related to acquisition of 5-fluorouracil resistance; however, its potential predictive value of response to preoperative chemoradiotherapy in locally advanced rectal cancer remains unknown.
Methods Nighty-two patients diagnosed with locally advanced rectal cancer who were preoperatively treated with chemoradiotherapy were selected for this study. Moreover, microRNA-21 expression was quantified in formalin-fixed paraffin-embedded biopsies from this cohort, and the results obtained were correlated with clinical and molecular characteristics, pathological response, and outcome.
Results MicroRNA-21 was found overexpressed in 77.6 % cases, and significantly correlated with tumor grade after preoperative chemoradiotherapy (P=0.013) and with pathological response (P=0.013). The odds ratio of having miR-21 overexpression and not getting a respond to chemoradiotherapy resulted in 9.75 CI 2.24 to 42. Sensitivity, specificity, negative predictive values, and positive predictive value were 86.6, 60, 42.8, and 92 %, respectively. Multivariate analysis confirmed the clinical significance of miR-21 determining preoperative chemoradiotherapy response.
Conclusions MicroRNA-21 expression efficiently predicts preoperative chemoradiotherapy pathological response in locally advanced rectal cancer.

Keywords miR-21 · Prognosis · Rectal cancer · Chemoradiotherapy

Introduction

Rectal carcinoma accounts for approximately 28 % of all colorectal malignancies [1, 2]. The treatment of choice for

Electronic supplementary material The online version of this article (doi:10.1007/s00384-015-2231-9) contains supplementary material, which is available to authorized users.

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Published online: 08 May 2015

locally advanced rectal cancer (LARC) involves a multidisciplinary approach and consist in preoperative chemoradiotherapy (CRT) followed by total mesorectal excision (TME) surgery [3]. The superiority of preoperative over postoperative CRT regarding local control, treatment compliance, and toxicity profile was demonstrated after CAO/ARO/AIO-94 (Working Group of Surgical Oncology/Radiation Oncology/Medical Oncology of the German Cancer Society) trial [3]. Several studies using 10-year follow-up outcomes have confirmed these findings [4].

After the introduction of preoperative CRT, an important clinic pathological observation was the variety in tumor response, ranging from pathologic complete response (pCR; ypT0N0) to absence of tumor regression at all or even tumor progression during therapy [5, 6]. Unfortunately, 5-fluorouracil (5FU)-based CRT produces a complete pathologic response in only 8 to 14 % of the patients, and distant metastases develop in one-third of the cases after 5 years from diagnosis [3, 7, 8]. Moreover, patients who do not respond to preoperative CRT are exposed to unnecessary toxicities, and resection of their primary tumor is delayed.

Of interest, a number of postsurgical prognostic factors have been proposed [9–11]. However, CRT response cannot be clinically predicted despite some studies have tried to identify predictive factors [12]. Thus, the identification of molecular alterations with predictive value of response to CRT in rectal cancer would be of high relevance for clinicians. MicroRNAs (miRNAs) are a class of 18-27-nucleotide single-stranded RNA molecules that negatively regulate the expression of specific target genes at posttranscriptional level. Deregulation of microRNAs (miRs) in colorectal cancer has been associated with tumor diagnosis, prognosis, and response to therapies, indicating that they might be promising biomarkers in clinical application [13].

Multiple studies have reported the role of microRNA-21 (miR-21) regulating colorectal cancer (CRC) biology [14, 15]. It has been published the relevance of miR-21 as a screening, diagnostic, and prognosis biomarker in CRC [16, 17]. Moreover, in vitro and in vivo studies using CRC cell lines have described molecular mechanisms of resistance to 5-fluorouracil (5-FU)-based chemotherapy related with miR-21 overexpression [18, 19]. Furthermore, lack of benefit from adjuvant chemotherapy after CRC surgery was associated with overexpression of miR-21 [20]. However, the ability of miR-21 predicting preoperative CRT response in rectal cancer is a very relevant question that remains to be addressed.

We show here that miR-21 is deregulated in rectal cancer patients and that its preoperative expression levels predict pathological response. Therefore, miR-21 could be used to define a subgroup of rectal cancer patients who will not benefit from preoperative CRT avoiding complications such us therapy-related toxicities and surgery delays.

Materials and methods

Patient characteristics

Ninety-two patients with a histologic diagnosis of locally advanced rectal adenocarcinoma, who were treated with preoperative CRT between 2007 and 2013 at the University Hospital Fundación Jiménez Díaz, were retrospectively selected for this study. The end of follow-up period was May 2014. The median follow-up time was 15 months. All patients had an accurate preoperative locoregional staging accomplished with magnetic resonance (MRI) of the pelvis and/or transrectal ultrasound (TRUS). A full body computed tomography scan (FBCT) to exclude stage IV disease was performed in all participants. The patients received rule-based chemoradiotherapy regimens based on 5-FU, whether capecitabine 825 mg/m^2/day or 5-fluoracil 425 mg/m^2/day and underwent surgery after 6 to 8 weeks after CRT completion. All participants provided written consent for the analysis and tissue storage at Fundación Jiménez Díaz biobank. The institutional review boards of Fundación Jiménez Díaz University Hospital approved the study. The study was performed in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guideline [21].

Sample size calculation

Recommended sample size for our given population size with a level of confidence of 95 % and a margin of error or degree of accuracy of 5.0 % was 63 by using the power analysis and sample size (PASS) bioinformatics tool.

Tumor specimens and pathologic response

Tumor specimens were collected from Fundación Jimenez Diaz biobank. Surgical specimens were evaluated according to the College of American Pathologist protocol for invasive carcinomas (TNM, 7th ed). Two independent pathologists who were blinded to patient outcome evaluated tumor regression grade according to the modified Ryan classification which categorize tumors in four levels of response: complete response, moderate response, minimal response, and poor response. Complete response refers to no viable cancer cells and score 0; moderate response refers to single cells or small groups of cancer cells and score 1; minimal response refers to residual cancer outgrown by fibrosis and score 2; poor response refers to minimal or no tumor kill with extensive residual cancer and scores 3. According to clinical guidelines, all regression grade was assessed in the primary tumor [22].

RNA isolation

Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumor specimens using RecoverAll Total Nucleic Acid Isolation kit (Ambion) according to manufacturer's instructions. Total RNA was eluted and quantified using a NanoDrop Spectrophotometer.

Quantification of miRNA expression levels

Samples were reverse transcribed using the TaqManHMicroRNA Reverse Transcription Kit (Applied Biosystems), and mature miRNAs were quantified by quantitative real-time RT-PCR using TaqMan MicroRNA Assays (Applied Biosystems) specific for miR-21 (reference number: 000397) and U6B as internal control. TaqMan MicroRNA assays can detect as few as ten copies of transcript in a sample and show a dynamic range greater than seven logs. Reactions were performed on an Applied Biosystems 7500 Sequence Detection System with the following cycling conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C}{}_{T}$ method [23].

Statistical analysis

Statistical analyses were performed using SPSS v14.0 (SPSS Inc, Chicago, IL). To correlate miR-21 overexpression and clinic-pathological variables, the $\chi 2$ test (Fisher exact test), based on bimodal distribution of data, was applied. All reported *P* values are two-sided. Predictive effects were assessed to pathologic response. A cutoff for miR-21 expression was established using a receiver operating characteristic (ROC) curve area under the curve, and its 95 % confidence interval was calculated. To assess the potential usefulness of mir-21 as a predictive biomarker, we choose the cutoff point that gave us the best sensitivity and specificity to discriminate rectal cancer pathologic response. Following this criteria, downregulation

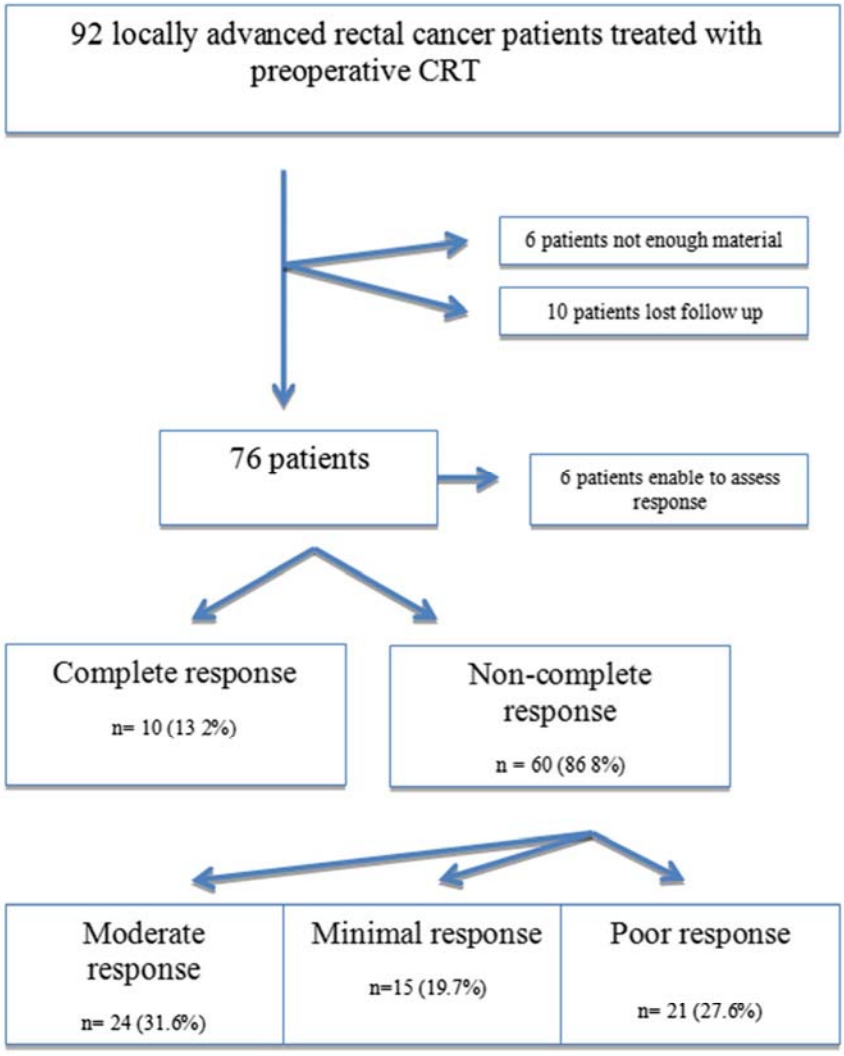

Fig. 1 Flowchart of patients through the study

Table 1 Baseline clinicopathological characteristics and association with preoperative CRT miR-21 expression levels

		No. cases (%)	No. miR-21 high %	No. miR-21 low %	*p*
miR-21 pre CRT		76 (100)	59 (77,6)	17 (22,4)	
Age	<60	23 (30)	17 (73)	6 (27)	
	>60	53 (70)	42 (79)	11 (21)	0.4081
Sex	Male	46 (60)	35 (76)	11 (24)	
	Female	30 (40)	24 (80)	6 (20)	0.4542
ECOG	0	42 (55)	34 (80)	8 (20)	
	1-II	34 (45)	25 (64)	9 (36)	0.3090
C-stage	I				
	II	4 (5)	2 (50)	2 (50)	
	III	71 (95)	56 (78)	15 (22)	0.2190
Neo adjuvant CRT	RT+5-FU	76 (100)	59 (77,6)	17 (22,4)	
Clinical stage post CRT	I	6 (25)	4 (66)	2 (34)	
	II	8 (33)	6 (75)	2 (25)	
	III	10 (41)	9 (90)	1 (10)	
	ND	24			0.5060
Adjuvant therapy	5-FU	57 (75)	43 (75)	14 (25)	
	FOLFOX	6 (7)	6 (100)	0	
	No	13 (18)	10 (76)	3 (24)	0.5452
Grade pre CRT	Low	20 (26)	15 (75)	5 (25)	
	High	48 (63)	40 (83)	8 (17)	
	ND	8 (11)	4 (50)	4 (50)	0.3134
ypT	ypT0	10 (13)	4 (40)	6 (60)	
	ypT1-2	35 (46)	29 (82)	6 (18)	
	ypT3-4	27 (35)	23 (92)	4 (8)	
	ypTx	4 (5)	2 (50)	2 (50)	**0.0150**
ypN	pN0	61 (80)	47 (77)	14 (23)	
	pN+	15 (20)	12 (80)	3 (20)	0.5555
Pathological stage	ypT0N0	10 (13)	4 (40)	6 (60)	
	ypI	31 (40)	26 (84)	5 (16)	
	ypII	21 (27)	17 (80)	4 (20)	
	ypIII	14 (18)	12 (85)	2 (15)	**0.0230**
Grade post CRT	No grade due to complete response	10 (13)	4 (40)	6 (60)	
	Low	20 (26)	18 (90)	2 (10)	
	Moderate-High	32 (42)	28 (87)	4 (13)	
	ND	14 (18)			**0.0130**
Pathology Response	Complete response	10 (13)	4 (40)	6 (60)	**0.0130**
	Moderate response	24 (31)	24 (100)	0	
	Minimal response	15 (19)	12 (80)	3(20)	
	Poor response	21 (27)	16 (77)	5 (23)	
	ND	6 (7)	3 (50)	3 (50)	
Recurrence	No	58 (76)	47 (61)	11 (64)	
	Yes	18 (24)	12 (39)	6 (36)	0.21
Death	No	70 (92)	56 (73)	14 (27)	
	Yes	6 (8)	3 (50)	3 (50)	0.22

ECOG Eastern Cooperative Oncology Group, *C-stage* preoperative/pretherapeutical TNM stage, *RT* radiotherapy, *5-FU* 5-flouracil, *pT* tumor size, *pN* lymph node, *ND* no data

was considered when miR-21 expression levels [$-\Delta C_T$] were lower than 2.8. PFS was calculated from the date of oncologic surgery to the date when progression was confirmed; OS was defined as the time elapsed from the date of diagnosis to the

date of cancer-related death. A P value <0.05 was considered statistically significant.

Results

MiR-21 expression in rectal cancer patients and its association with clinical and pathological characteristics

Ninety-two patients with LARC treated with preoperative CRT were included in this study. A flowchart diagram of the cohort is shown in Fig. 1. From 6 patients, there was not enough tissue available, and 10 patients were excluded due to lost of clinical follow-up data. Complete response was observed in 13.2 % of patients, and cases with noncomplete response included those that achieved a moderate response (31.6 %), a minimal response (19.7 %), and a poor response (27.6 %). Baseline clinic pathological characteristics of the cohort and its association with miR-21 expression are shown in Table 1.

The prevalence of miR-21 overexpression was 77.6 % cases (59 out of 76 cases). To investigate the potential value of miR-21 predicting response to preoperative CRT, patients with complete response and patients without complete response were compared. Interestingly, pathologic response was significantly associated with miR-21 overexpression (P= 0.013). Furthermore, tumor grade before preoperative CRT was not associated with miR-21 overexpression. However, tumor grade showed a significant association with miR-21 levels after preoperative CRT (P=0.013), which is consistent with the association between miR-21 and pathologic response. The down sizing effect of the preoperative CRT is shown in Supplementary Table 2. Of note, 85 out of the 92 patients with LARC selected for the study had complete data for both pretherapeutical and posttherapeutical TNM stage. None of the patients had a pretherapeutical stage 0 or I, and there were 4 (4.7 %) with stage II and 81 (95.3 %) with stage III. On the other hand, 17 cases (20 %) showed no residual tumor posttherapeutically, and 31 (36.5 %), 20 (23.5 %), and 17 patients (20 %) had stage I, II, III, respectively.

Since miR-21 expression correlates with tumor grade and stage, we analyzed the correlation between both clinical parameters (Supplementary Table 2). We observed that 67 out of 92 patients had complete data for both posttherapeutical TNM stage and grade. Interestingly, 10 cases had no tumor grade and no tumor TNM stage because a complete response was obtained. Moreover, there were no patients with stage I having a high tumor grade (G3), and most of cases with low tumor grade (G1) were stage I. In contrast, patients with TNM stages II and III were found associated with higher tumor grades (G2 and G3).

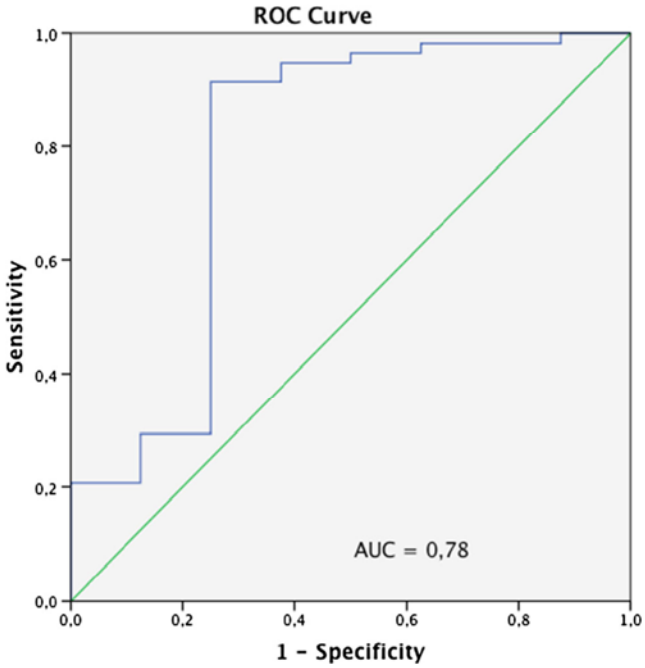

Fig. 2 ROC curve showing the potential usefulness of miR-21 as a predictive biomarker for preoperative chemoradiotherapy

Table 2 Preoperative CRT miR-21 predictive value

Noncomplete response vs complete response			
	-	+	Total
miR-21 low	8	6	14
miR-21 high	52	4	56
Total	60	10	70
PPV (%) 92		Sensitivity (%) 86,6	
NPV (%) 42,8		Specificity (%) 60	

Legend: "+" pathological complete response; "-"=moderate, minimal, or poor response; miR-21 high=miR-21>2,8 (cutoff derived by receive operating characteristic curve); miR-21 low=miR-21<2,8 (cutoff derived by receive operating characteristic curve); *PPV* positive predictive value, *NPV* negative predictive value

Table 3 Multivariable logistic analysis for preoperative CRT miR-21 expression and diagnosis factors in complete responder and noncomplete responder patients

Noncomplete response vs complete response		
	OR (95 % CI)	*p*
Age, < 60 vs>60	1,34 (0,43 to 4,22)	0,4
Gender, Female vs Male	1,25 (0,41 to 3,86)	0,45
ECOG, O vs I-II	0,65 (0,22 to 1,93)	0,31
Grade pre CRT, high-moderate vs low	1,66 (0,47 to 5,9)	0,31
Clinical stage, II vs III	3,73 (0,48 to 28,7)	0,22
miR-21 high vs low	9,75 (2,24 to 42)	0,01

CI confidence interval, *OR* odds ratio, *miR-21 high* miR-21>2,8 (cutoff derived by receive operating characteristic curve), *miR-21 low* miR-21< 2,8 (cutoff derived by receive operating characteristic curve)

MiR-21 has predictive value of pathological response to CRT in LARC patients

To assess the potential usefulness of miR-21 as a predictive biomarker, ROC curves were generated. MiR-21 expression levels yielded an area under the curve (AUC) value of 0.78 (95 % confidence interval [CI]=0.56 to 0.99), with 86.6 % sensitivity and 60 % specificity in distinguishing rectal cancer from complete response to noncomplete response. Thus, ROC curve revealed that preoperative miR-21 levels were robust in discriminating responders and nonresponders. Positive predictive value (PPV) was 92 %, and negative predictive value (NPV) was 42.8 % (Fig. 2 and Table 2). In addition, multivariable logistic regression analyses showed that preoperative CRT miR-21 could be a predictive pathologic response tool. The odds ratio for noncomplete response was 9.75 (95 % CI=2.24 to 42) (Table 3).

Discussion

Although previous studies have shown the potential value of some gene signatures as well as microRNA expression profiles to predict preoperative CRT response in locally advanced rectal cancer [24–27], these genes are not well established to use in current clinical routine. Here, we report that miR-21 has a potential value predicting pathologic response in samples of patients with LARC before preoperative CRT. Individualized treatment selection for patients with rectal cancer relies on preoperative clinical variables. Considering that more than 50 % of the patients do not respond to preoperative CRT therapy and the lack of any clinicopathological tool to guide preoperative treatment, novel predictive biomarkers are needed to avoid toxicity treatments and surgery delays. In concordance with our findings, it has been recently demonstrated that forced expression of miR-21 significantly increased the resistance of tumor cells to 5-FU and radiation therapy in HT-29 colon cancer cells [19]. Moreover, the use of xenograft models has shown that miR-21 overexpression dramatically reduces the therapeutic efficacy of 5-FU [18].

Several studies have reported microRNAs such as miR-16, miR-590-5p, miR-153, miR-519c-3p, and miR-561, which are associated with CRT response, but these works did not include miR-21 [28, 29]. However, changes in microRNA expression profiles were found in 35 patients with rectal carcinoma comparing pretherapeutic tissue with biopsies taken 2 weeks after treatment. Although miR-21, miR-10a, miR-145, miR-212, miR-339, and miR-361 were found to vary after therapy, only miR-125b and miR-137 had a positive correlation with a poorer tumor regression response [30]. Another study comparing pretherapeutic and posttherapeutic samples from 40 patients with LARC showed lower miR-21 levels in posttherapeutic tissues [31]. However, they did not found statistical significance association between miR-21 overexpression and pathologic response to treatment probably due to the small size of the cohort studied. Furthermore, miR-21 expression levels were found upregulated in rectal cancer patients treated with local excision compared with normal paired mucosa but no differences between tumor tissue when comparing recurrence patients and nonrecurrence patients. Of importance, patients in this study did not receive preoperative CRT [32]. Moreover, it has been recently reported that miR-21 and SATB1 could predict complete response to preoperative CRT in rectal cancer patients. Despite that it is a well-conducted study showing very interesting findings, the relative short number of cases included in the training (n=27) and validating groups (n=16) makes necessary to further investigate the miR-21 prognostic value [33]. Finally, recent works highlight the potential clinical significance of circulating miR-21 in colorectal cancer patients [34, 35].

Although the clinical significance of tumor pathologic response is still under investigation, several studies have suggested that the degree of tumor regression may be of high clinical relevance because it could be used as a treatment monitoring and prognostic parameter [36–38]. However, pathologic response was not associated to OS and PFS in our cohort, probably due to the limitation of the short median time of follow-up period that the patients had in our study (15 months) compromising OS and PFS analyses. Considering the median PFS and OS that currently locally advanced rectal cancer patients treated with surgery and CRT achieved, a reanalysis using updated data would be of interest in the next 2 to 5 years.

In conclusion, previous studies suggested a potential role of miR-21 predicting response to therapy in rectal cancer. However, this question remained to be fully clarified. We analyze in this study the miR-21 expression in 76 rectal adenocarcinomas in FFPE tissue before preoperative CRT. Interestingly, this is the largest study evaluating miR-21 as a predictor of response to therapy to date, and we show that miR-21 is able to determine response to neoadjuvant CRT in LARC patients. However, an experimental validation of these results using the same cutoff value in an independent patient cohort remains necessary to further confirm our findings. Moreover, more studies are warranted to further determine the biological and clinical significance of miR-21 in rectal cancer.

Acknowledgments This study was supported by PI12/01552 and PI13/02609 grants from Instituto de Salud Carlos III FEDER and S2010/BMD2344 grant from the Comunidad de Madrid. The authors declare that they have no conflict of interest.

References

1. Rodel C, Hofheinz R, Liersch T (2012) Rectal cancer: state of the art in 2012. Curr Opin Oncol 24:441–447
2. Brenner H, Kloor M, Pox CP (2014) Colorectal cancer. Lancet 383: 1490–1502

3. Sauer R, Becker H, Hohenberger W, Rodel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R, German Rectal Cancer Study G (2004) Preoperative versus postoperative chemoradiotherapy for rectal cancer. N Engl J Med 351:1731–1740
4. Mamon HJ (2012) Long-term follow-up of a paradigm-changing study: the paradigm still holds. J Clin Oncol 30:1901–1903
5. Chetty R, Gill P, Govender D, Bateman A, Chang HJ, Deshpande V, Driman D, Gomez M, Greywoode G, Jaynes E, Lee CS, Locketz M, Rowsell C, Rullier A, Serra S, Shepherd N, Szentgyorgyi E, Vajpeyi R, Wang LM, Bateman A (2012) International study group on rectal cancer regression grading: interobserver variability with commonly used regression grading systems. Hum Pathol 43:1917–1923
6. Chetty R, Gill P, Bateman AC, Driman DK, Govender D, Bateman AR, Chua YJ, Greywoode G, Hemmings C, Imat I, Jaynes E, Lee CS, Locketz M, Rowsell C, Rullier A, Serra S, Szentgyorgyi E, Vajpeyi R, Delaney D, Wang LM (2012) Pathological grading of regression: an International Study Group perspective. J Clin Pathol 65:865–866
7. Bosset JF, Collette L, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bardet E, Beny A, Ollier JC, Trial ERG (2006) Chemotherapy with preoperative radiotherapy in rectal cancer. N Engl J Med 355:1114–1123
8. Gerard JP, Conroy T, Bonnetain F, Bouche O, Chapet O, Closon-Dejardin MT, Untereiner M, Leduc B, Francois E, Maurel J, Seitz JF, Buecher B, Mackiewicz R, Ducreux M, Bedenne L (2006) Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: results of FFCD 9203. J Clin Oncol 24:4620–4625
9. Smith FM, Reynolds JV, Miller N, Stephens RB, Kennedy MJ (2006) Pathological and molecular predictors of the response of rectal cancer to neoadjuvant radiochemotherapy. Eur J Surg Oncol 32:55–64
10. Nagtegaal ID, Gosens MJ, Marijnen CA, Rutten HJ, van de Velde CJ, van Krieken JH (2007) Combinations of tumor and treatment parameters are more discriminative for prognosis than the present TNM system in rectal cancer. J Clin Oncol 25:1647–1650
11. Quirke P, Steele R, Monson J, Grieve R, Khanna S, Couture J, O'Callaghan C, Myint AS, Bessell E, Thompson LC, Parmar M, Stephens RJ, Sebag-Montefiore D, MCN-CCT Investigators and NCCS Group (2009) Effect of the plane of surgery achieved on local recurrence in patients with operable rectal cancer: a prospective study using data from the MRC CR07 and NCIC-CTG CO16 randomised clinical trial. Lancet 373:821–828
12. Casado E, Garcia VM, Sanchez JJ, Blanco M, Maurel J, Feliu J, Fernandez-Martos C, de Castro J, Castelo B, Belda-Iniesta C, Sereno M, Sanchez-Llamas B, Burgos E, Garcia-Cabezas MA, Mancenido N, Miquel R, Garcia-Olmo D, Gonzalez-Baron M, Cejas P (2011) A combined strategy of SAGE and quantitative PCR Provides a 13-gene signature that predicts preoperative chemoradiotherapy response and outcome in rectal cancer. Clin Cancer Res 17:4145–4154
13. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M (2012) microRNAs in cancer management. Lancet Oncol 13:e249–e258
14. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128–2136
15. Yamamichi N, Shimomura R, Inada K, Sakurai K, Haraguchi T, Ozaki Y, Fujita S, Mizutani T, Furukawa C, Fujishiro M, Ichinose M, Shiogama K, Tsutsumi Y, Omata M, Iba H (2009) Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. Clin Cancer Res 15:4009–4016
16. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 299:425–436
17. Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A (2013) Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst 105:849–859
18. Valeri N, Gasparini P, Braconi C, Paone A, Lovat F, Fabbri M, Sumani KM, Alder H, Amadori D, Patel T, Nuovo GL, Fishel R, Croce CM (2010) MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). Proc Natl Acad Sci U S A 107:21098–21103
19. Deng J, Lei W, Fu JC, Zhang L, Li JH, Xiong JP (2014) Targeting miR-21 enhances the sensitivity of human colon cancer HT-29 cells to chemoradiotherapy in vitro. Biochem Biophys Res Commun 443:789–795
20. Oue N, Anami K, Schetter AJ, Moehler M, Okayama H, Khan MA, Bowman ED, Mueller A, Schad A, Shimomura M, Hinoi T, Aoyagi K, Sasaki H, Okajima M, Ohdan H, Galle PR, Yasui W, Harris CC (2014) High miR-21 expression from FFPE tissues is associated with poor survival and response to adjuvant chemotherapy in colon cancer. Int J Cancer 134:1926–1934
21. Altman DG, McShane LM, Sauerbrei W, Taube SE (2012) Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. PLoS Med 9, e1001216
22. Greenson JK, Bonner JD, Ben-Yzhak O, Cohen HI, Miselevich I, Resnick MB, Trougouboff P, Tomsho LD, Kim E, Low M, Almog R, Rennert G, Gruber SB (2003) Phenotype of microsatellite unstable colorectal carcinomas: Well-differentiated and focally mucinous tumors and the absence of dirty necrosis correlate with microsatellite instability. Am J Surg Pathol 27:563–570
23. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108
24. Ghadimi BM, Grade M, Difilippantonio MJ, Varma S, Simon R, Montagna C, Fuzesi L, Langer C, Becker H, Liersch T, Ried T (2005) Effectiveness of gene expression profiling for response prediction of rectal adenocarcinomas to preoperative chemoradiotherapy. J Clin Oncol 23:1826–1838
25. Kim IJ, Lim SB, Kang HC, Chang HJ, Ahn SA, Park HW, Jang SG, Park JH, Kim DY, Jung KH, Choi HS, Jeong SY, Sohn DK, Kim DW, Park JG (2007) Microarray gene expression profiling for predicting complete response to preoperative chemoradiotherapy in patients with advanced rectal cancer. Dis Colon Rectum 50:1342–1353
26. Rimkus C, Friederichs J, Boulesteix AL, Theisen J, Mages J, Becker K, Nekarda H, Rosenberg R, Janssen KP, Siewert JR (2008) Microarray-based prediction of tumor response to neoadjuvant radiochemotherapy of patients with locally advanced rectal cancer. Clin Gastroenterol Hepatol 6:53–61
27. Folkvord S, Flatmark K, Dueland S, de Wijn R, Groholt KK, Hole KH, Nesland JM, Ruijtenbeek R, Boender PJ, Johansen M, Giercksky KE, Ree AH (2010) Prediction of response to preoperative chemoradiotherapy in rectal cancer by multiplex kinase activity profiling. Int J Radiat Oncol Biol Phys 78:555–562
28. Kheirelseid EA, Miller N, Chang KH, Curran C, Hennessey E, Sheehan M, Newell J, Lemetre C, Balls G, Kerin MJ (2013) miRNA expressions in rectal cancer as predictors of response to neoadjuvant chemoradiation therapy. Int J Colorectal Dis 28:247–260
29. Della Vittoria Scarpati G, Falcetta F, Carlomagno C, Ubezio P, Marchini S, De Stefano A, Singh VK, D'Incalci M, De Placido S, Pepe S (2012) A specific miRNA signature correlates with complete pathological response to neoadjuvant chemoradiotherapy in

locally advanced rectal cancer. Int J Radiat Oncol Biol Phys 83: 1113–1119

30. Svoboda M, Sana J, Fabian P, Kocakova I, Gombosova J, Nekvindova J, Radova L, Vyzula R, Slaby O (2012) MicroRNA expression profile associated with response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer patients. Radiat Oncol 7:195
31. Drebber U, Lay M, Wedemeyer I, Vallbohmer D, Bollschweiler E, Brabender J, Monig SP, Holscher AH, Dienes HP, Odenthal M (2011) Altered levels of the onco-microRNA 21 and the tumor-supressor microRNAs 143 and 145 in advanced rectal cancer indicate successful neoadjuvant chemoradiotherapy. Int J Oncol 39: 409–415
32. Riordan AM, Thomas MK, Ronnekleiv-Kelly S, Warner T, Geiger PG, Kennedy GD (2012) Utility of micro-ribonucleic acid profile for predicting recurrence of rectal cancer. J Surg Res 177:87–92
33. Lopes-Ramos C, Habr-Gama A, Quevedo B, Felício N, Bettoni F, Koyama F, Asprino P, Galante P, Gama-Rodrigues J, Camargo A, Perez R, Parmigiani R (2014) Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients. BMC Med Genomics 7:68
34. Monzo M, Martínez-Rodenas F, Moreno I, Navarro A, Santasusagna S, Macias I, Muñoz C, Tejero R, Hernández R (2015) Differential MIR-21 Expression in Plasma From Mesenteric Versus Peripheral Veins: An Observational Study of Disease-free Survival in Surgically Resected Colon Cancer Patients. Medicine (Baltimore) 94, e145
35. Shan L, Ji Q, Cheng G, Xia J, Liu D, Wu C, Zhu B, Ding Y (2015) Diagnostic value of circulating miR-21 for colorectal cancer: A meta-analysis. Cancer Biomark 15:47–56
36. Rodel C, Martus P, Papadoupolos T, Fuzesi L, Klimpfinger M, Fietkau R, Liersch T, Hohenberger W, Raab R, Sauer R, Wittekind C (2005) Prognostic significance of tumor regression after preoperative chemoradiotherapy for rectal cancer. J Clin Oncol 23:8688–8869
37. Suarez J, Vera R, Balen E, Gomez M, Arias F, Lera JM, Herrera J, Zazpe C (2008) Pathologic response assessed by Mandard grade is a better prognostic factor than down staging for disease-free survival after preoperative radiochemotherapy for advanced rectal cancer. Colorectal Dis 10:563–568
38. Park IJ, You YN, Agarwal A, Skibber JM, Rodriguez-Bigas MA, Eng C, Feig BW, Das P, Krishnan S, Crane CH, Hu CY, Chang GJ (2012) Neoadjuvant treatment response as an early response indicator for patients with rectal cancer. J Clin Oncol 30:1770–1776

Article 7: MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer.[7]

International Journal of *Molecular Sciences*

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Article

MicroRNA-31 Emerges as a Predictive Biomarker of Pathological Response and Outcome in Locally Advanced Rectal Cancer

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Academic Editor: William Chi-shing Cho
Received: 22 April 2016; Accepted: 27 May 2016; Published: 3 June 2016

Abstract: Neoadjuvant chemoradiotherapy (CRT) followed by total mesorectal excision has emerged as the standard treatment for locally advanced rectal cancer (LARC) patients. However, many cases do not respond to neoadjuvant CRT, suffering unnecessary toxicities and surgery delays. Thus, identification of predictive biomarkers for neoadjuvant CRT is a current clinical need. In the present study, microRNA-31 expression was measured in formalin-fixed paraffin-embedded (FFPE) biopsies from 78 patients diagnosed with LARC who were treated with neoadjuvant CRT. Then, the obtained results were correlated with clinical and pathological characteristics and outcome. High microRNA-31 (miR-31) levels were found overexpressed in 34.2% of cases. Its overexpression significantly predicted poor pathological response (p = 0.018) and worse overall survival (OS) (p = 0.008). The odds ratio for no pathological response among patients with miR-31 overexpression was 0.18 (Confidence Interval = 0.06 to 0.57; p = 0.003). Multivariate analysis corroborated the clinical impact of miR-31 in determining pathological response to neoadjuvant CRT as well as OS. Altogether, miR-31 quantification emerges as a novel valuable clinical tool to predict both pathological response and outcome in LARC patients.

Keywords: miR-31; neoadjuvant chemoradiotherapy; rectal cancer; prognosis

1. Introduction

Colorectal carcinoma (CRC) incidence, morbidity, and mortality rates vary markedly around the world, and rectal carcinoma represents approximately 28% of all CRC [1]. The standard of care for locally advanced rectal cancer (LARC) involves a multidisciplinary approach consisting of neoadjuvant chemoradiotherapy (CRT) followed by total mesorectal excision (TME) surgery. This fact was established after several studies showing that CRT before total mesorectal excision (neoadjuvant chemoradiotherapy) was associated with a lower percentage of patients with local recurrence than either with total mesorectal excision surgery followed by adjuvant CRT or total mesorectal excision surgery alone [2,3]. After the initiation of neoadjuvant CRT, a relevant observation is the range of pathological tumor response, from cases achieving a pathological complete response (pCR; ypT0N0) to others getting an absence of tumor regression at all or even tumor progression during therapy [4,5]. Of importance, pCR detected by pathological examination of the resected specimen is associated with significantly better prognosis as compared with patients with residual tumor, particularly residual nodal disease. Similarly, minimal residual disease is associated with better prognosis than gross residual disease [6,7]. Currently, the neoadjuvant CRT regimen most widely used is based on fluoropyrimidines concurrent with conventional fractionation radiotherapy [2]. Nevertheless, more than one-third of cases develop distant metastasis within 10 years from diagnosis and complete pathological response only occurs in 8% to 14% of patients. Thus, those patients who do not respond suffer undesired toxicities and delays in the resection of the primary tumor [8,9].

There are currently no effective methods to predict which patients will respond to neoadjuvant CRT. Despite some prognostic factors of local recurrence, distant recurrence and outcome have been proposed after tumor mesorectal excision surgery [10–12]; neoadjuvant CRT predictive biomarkers with an impact in recurrence and outcome are not still established in clinical practice [12–14]. The identification of patients who have a higher possibility of responding to preoperative CRT could be important in improving survival and decreasing treatment morbidity and local control in LARC. Moreover, patients who are potential non-responders could be moved to alternative therapeutic strategies. Therefore, the identification of novel alterations with predictive value of response to neoadjuvant CRT would be of high relevance for an optimal multidisciplinary treatment approach in this LARC patient subgroup.

MicroRNAs (miRNAs) are short (18–27 nucleotides) non-coding single-stranded RNA molecules which negatively regulate the expression of specific target genes at the post-transcriptional level. Depending on their target genes, microRNA deregulation has been related to CRC development, progression, or therapy response. Moreover, they can be easily detected in rectal cancer tissue and blood, so they have been proposed as promising biomarkers for diagnosis, prognosis, and monitoring therapies in this disease [15–17]. MicroRNA-31 (miR-31) maps on 9p21.3 and is one of the most significantly deregulated miRNAs in rectal cancer. It has been reported to be overexpressed in CRC cell lines as well as in rectal tumor tissue compared with the normal paired rectal mucosa [18]. In functional studies, the inhibition of miR-31 is able to impair CRC cell proliferation, invasion, and promote apoptosis—which suggests its oncogenic role in this disease [19]. Furthermore, high miR-31 levels have been described to correlate with tumor stage [18,20], poor prognosis [21], and shorter progression-free survival in CRC patients treated with anti-epithelial growth factor receptor (anti-EGFR) therapies [21]. Of importance, miR-31 suppression increases sensitivity to 5-fluorouracil (5-FU) and affects cell migration and invasion in the CRC cell line HCT-116 [22]. However, miR-31 ability to predict neoadjuvant CRT pathological response and outcome in LARC is a relevant question that remains to be explored. Here, we report that miR-31 deregulation is a common event in LARC that determines poor outcome. Moreover, this alteration is of high therapeutic relevance because it defines a subgroup of LARC patients who will not respond to neoadjuvant CRT.

2. Results

2.1. Prevalence of miR-31 Deregulation in Locally Advanced Rectal Cancer (LARC) and Its Relation with Pathological and Clinical Characteristics

Eighty-two patients with LARC treated with neoadjuvant CRT were selected. All of them had a minimal follow-up greater than three years. The cohort flowchart diagram is shown in Figure 1. Owing to the loss of clinical follow-up data, four patients were excluded. Complete response, moderate response, minimal response, and a poor response was observed in 12.3%, 37%, 18.3%, and 28.4% of patients, respectively.

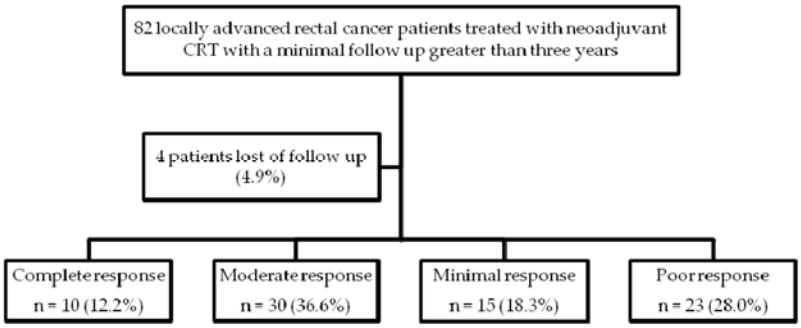


Figure 1. Patients flowchart through the study. CRT: chemoradiotherapy.

High miR-31 levels were observed in 34.2% of cases (27 out of 78). To explore the potential value of miR-31 predicting response to neoadjuvant CRT, patients with different grades of pathological response were compared. Interestingly, those patients who developed complete response tended to have low microRNA-31 levels, whereas the subgroup of patients with a poor pathological response showed high levels of miR-31. (p = 0.018). No statistical significance was observed in age, sex, Eastern Cooperative Oncology Group (ECOG) scale of performance status, histological grade, clinical stage, tumor size, and lymph node stage between patients with high and low miR-31 expression levels (Table 1). Similarly, no differences were found for pathological stage and downstaging.

Table 1. Baseline clinical and pathological characteristics of our locally advanced rectal cancer (LARC) cohort and its relation with miR-31 expression.

Clinical and pathological characteristics		n° Cases (%)	n° miR-31 High [1] (%)	n° miR-31 Low [2] (%)	p
miR-31 [3] [pre] CRT		78 (100%)	27 (34.2)	51 (65.8)	
Age	<60	24 (30.7)	10 (41.6)	14 (58,4)	0.267
	>60	54 (69.3)	17 (31.4)	37 (68.6)	
Sex	Male	47 (60)	15 (31.9)	32 (68.1)	0.454
	Female	31 (40)	8 (68.1)	19 (31.9)	
ECOG [4]	0	49 (62.8)	14 (28.5)	35 (71.5)	0.113
	I	29 (37.2)	13 (71.5)	16 (28.5)	
Clinical stage pre CRT [5]	II	4 (5.2)	3 (75)	1 (25)	0.121
	III	73 (94.8)	24 (30.7)	49 (69.3)	
Neoadjuvant CRT	RT + 5-FU [6] based	78 (100%)			
Adjuvant therapy	5-FU	55 (70.5)	20 (36.4)	35 (63.6)	0.407
	FOLFOX [7]	6 (7.7)	3 (50)	3 (50)	
	Other	17 (21.8)	4 (23.5)	13 (76.5)	
Grade pre CRT	Low	20 (25.8)	5 (25)	15 (75)	0.410
	High	50 (64)	20 (40)	30 (60)	
	ND [8]	8 (10.2)	2 (25)	6 (75)	

Table 1. *Cont.*

Clinical and pathological characteristics		n° Cases (%)	n° miR-31 High [1] (%)	n° miR-31 Low [2] (%)	*p*
ypT [9]	ypT0	10 (12.8)	1 (10)	9 (90)	0.372
	ypT1-2	33 (42.4)	13 (39.4)	20 (60.6)	
	ypT3-4	32 (41)	12 (37.5)	20 (62.5)	
	ypTx	3 (3.8)	1 (33.3)	2 (66.7)	
ypN [10]	pN0	61 (78.2)	18 (29.5)	43 (70.5)	0.086
	pN+	17 (21.8)	9 (52.9)	8 (47.1)	
Pathological stage	ypT0N0	10 (12.8)	1 (10)	9 (90)	0.133
	ypI	30 (38.5)	11 (36.6)	19 (63.4)	
	ypII	21 (27)	6 (28.6)	15 (71.4)	
	ypIII	17 (21.7)	9 (52.9)	8 (47.1)	
Downstaging	No	19 (24.3)	9 (47.4)	10 (52.6)	0.143
	Yes	59 (75.7)	18 (52.6)	41 (47.4)	
Pathology response	Complete response	10 (12.8)	1 (10)	9 (90)	0.018
	Moderate response	30 (38.5)	7 (23.3)	23 (76.7)	
	Minimal response	15 (19.2)	5 (33.3)	10 (66.7)	
	Poor response	23 (29.5)	14 (60.8)	9 (39.2)	

[1] miR-31 high = ΔC_T miR-31 < 0.34 (cut-off established by a receiver operating characteristic (ROC) curve); [2] miR-31 low = ΔC_T miR-31 > 0.34 (cut-off established using a ROC curve); [3] miR-31 = microRNA 31; [4] ECOG = Eastern Cooperative Oncology Group; [5] CRT = Chemoradiotherapy; [6] 5-FU = 5-fluorouracil; [7] FOLFOX = Fluorouracil, Oxaliplatin, Leucovorin; [8] ND = No data; [9] ypT = tumor size after CRT; [10] ypN = pathological lymph node after CRT.

2.2. *miR-31 Deregulation Predicts Pathological Response to Neoadjuvant Chemoradiotherapy (CRT) in LARC Patients*

Receiver operating characteristic (ROC) curves were generated in order to investigate the potential utility of miR-31 as a predictive biomarker of response to neoadjuvant CRT. miR-31 expression levels provide an area under the curve (AUC) value of 0.71 (95% confidence interval (CI) = 0.57 to 0.84; $p = 0.001$) with 60.8% sensitivity and 76.3% specificity in distinguishing rectal cancer from poor responders to minimal, moderate, and complete responders. Negative predictive value (NPV) was 82.3% and positive predictive value (PPV) was 51.8% (Figure 2 and Table 2).

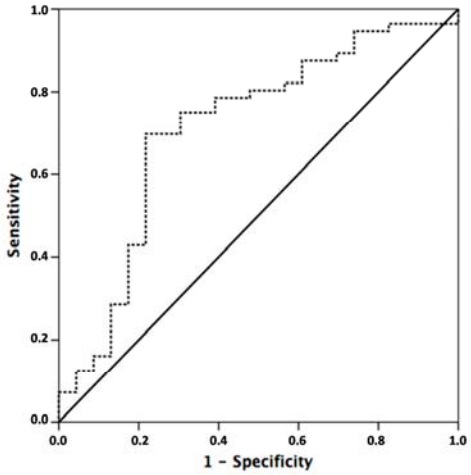


Figure 2. Receiver operating characteristic (ROC) Curve. Based on this ROC curve, the cutoff point that provides the best sensitivity and specificity to separate rectal cancer from any response to absence of response (Ryan 3) was selected. According with this criteria, miR-31 high (or overexpressed) was defined as ΔC_T miR-31 < 0.34 and miR-31 low was defined as ΔC_T miR-31 > 0.34. The dash line represents the coordinated points of the ROC curve. The solid line represents the ROC curve diagonal reference line.

Table 2. MiR-31 predicts pathological response to neoadjuvant CRT.

Responders *vs.* Non-Responders			
Response	**Response** [1]	**Non-Response** [2]	**Total**
miR-31 high [3]	13	14	27
miR-31 low [4]	42	9	51
Total	55	23	78
NPV [5] (%) 82.3		Specificity (%) 76.3	
PPV [6] (%) 51.8		Sensitivity (%) 60.8	

[1] "Response" = moderate, minimal, or complete response; [2] "Non-Response" = poor pathological response; [3] miR-31 high = ΔC_T miR-31 < 0.34 (cutoff derived by receive operating characteristic curve); [4] miR-31 low= ΔC_T miR-31 > 0.34 (cutoff derived by receive operating characteristic curve); [5] NPV = negative predictive value; [6] PPV = positive predictive value.

Moreover, multivariable logistic regression analyses revealed that miR-31 expression levels measured before the beginning of neoadjuvant CRT is a predictive pathological response marker. The odds ratio for non-responders was 0.18 (95% CI = 0.06 to 0.57; $p = 0.003$) (Table 3).

Table 3. Multivariable logistic analysis for miR-31 expression measured pre neoadjuvant CRT and the rest of clinic-pathological factors measured before neoadjuvant CRT in responder and non-responder patients.

Responders [1] ***vs.* Non-Responders** [2]	[3] **OR (95% CI** [4]**)**	***p***
Age, <60 *vs.* >60	0.97 (0.30 to 3.06)	0.962
Gender, Female *vs.* Male	1.25 (0.41 to 3.86)	0.452
ECOG [5], O *vs.* I	0.79 (0.25 to 2.51)	0.799
Grade pre CRT [6], high-moderate *vs.* low	1.25 (0.53 to 2.96)	0.600
Clinical stage, II *vs.* III	2.51 (0.20 to 30.37)	0.468
miR-31 high [7] *vs.* low [8]	0.18 (0.06 to 0.57)	0.003

[1] "*Response*" = moderate, minimal or complete response; [2] "*Non-Response*" = poor pathological response; [3] OR = Odds ratio; [4] CI = Confidence interval; [5] ECOG = Eastern Cooperative Oncology Group; [6] CRT = chemoradiotherapy; [7] miR-31 high = ΔC_T miR-31 < 0.34 (cutoff derived by receive operating characteristic curve); [8] miR-31 low= ΔC_T miR-31 > 0.34 (cutoff obtained by a receive operating characteristic curve).

2.3. High miR-31 Levels Determines Poor Outcome in LARC Patients Treated with Neoadjuvant CRT

To further evaluate the clinical significance of miR-31 in LARC, we next investigated whether miR-31 levels could serve as a predictor of patient outcome. Interestingly, patients with higher miR-31 levels had statistically significantly worse overall survival ($p = 0.008$, Figure 3A). Despite high miR-31 expression also associated with shorter disease-free survival, significance was not achieved ($p = 0.070$)—probably due to the small number of cases included in this cohort (Figure 3B).

Additionally, Cox proportional hazard regression analyses showed that in the univariate analysis, poor outcome in LARC patients was related to high levels of miR-31 (Hazard Ratio (HR) = 5.077; 95% CI = 1.366 to 18.863; $p = 0.015$) and pathological stage (HR = 1.890; 95% CI = 1.027 to 3.478; $p = 0.043$). In the same way, multivariable analysis proved that high levels of miR-31 can be used as an independent prognostic biomarker for determining outcome in LARC patients treated with neoadjuvant CRT (HR = 0.206; 95% CI = 0.051 to 0.840; $p = 0.028$) (Table 4).

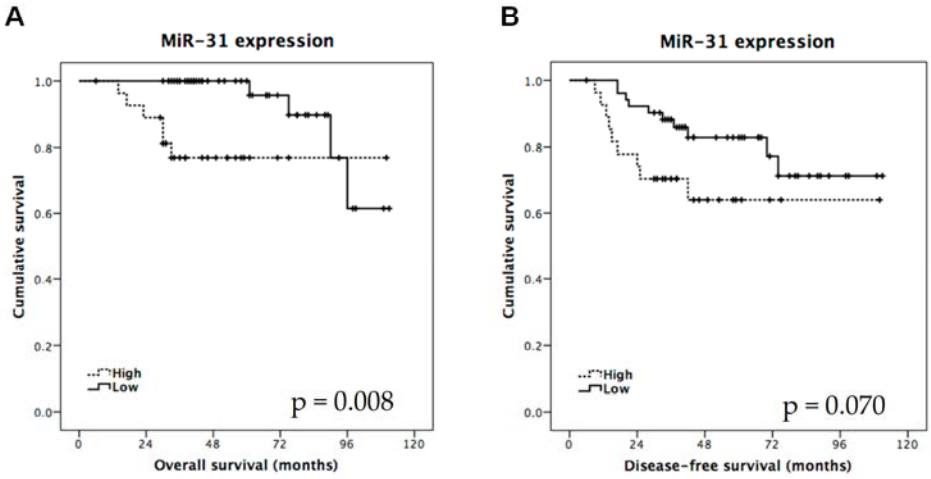


Figure 3. Kaplan-Meier analysis of (**A**) overall survival (OS) and (**B**) disease-free survival (DFS) in LARC patients based upon miR-31 expression in primary tumors measured prior to the neoadjuvant treatment.

Table 4. LARC patients factors predictive of poor overall survival: Univariate and multivariable analyses.

Variables	Univariate		Multivariate	
	HR [1] (95% CI [2])	*p*	HR (95% CI)	*p*
Age, >60 *vs.* <60	0.915 (0.246 to 3.407)	0.895		
Sex, female *vs.* male	1.024 (0.56 to 1.86)	0.928		
Clinical stage, II *vs.* III	2.229 (0.274 to 18.132)	0.453		
Pathological ypT [3], ypT0 *vs.* ypT1/T2 *vs.* ypT3/T4	2.064 (0.214 to 19.902)	0.531		
Pathological ypN [4], N+ *vs.* N−	2.907 (0.918 to 9.211)	0.070		
Pathological stage	1.890 (1.027 to 3.478)	0.043	2.411 (1.136 to 5.114)	0.022
Pathological response, poor *vs.* complete *vs.* moderate *vs.* minimal	1.346 (0.70 to 2.56)	0.366		
MiR-31 expression, high [5] ($\Delta C_T < 0.34$) *vs.* low [6] ($\Delta C_T > 0.34$)	5.077 (1.366 to 18.863)	0.015	0.206 (0.051 to 0.840)	0.028

[1] HR = Hazard ratio; [2] CI = Confidence Interval; [3] ypT = tumor size after CRT; [4] ypN = pathological lymph node after CRT; [5] miR-31 high = ΔC_T miR-31 < 0.34 (cutoff derived by receive operating characteristic curve); [6] miR-31 low = ΔC_T miR-31 > 0.34 (cutoff derived by receive operating characteristic curve).

3. Discussion

Personalized treatment selection for patients with LARC relies on clinical variables measured prior to neoadjuvant CRT. Taken into account that, depending on the series, between 45% and 20% of the patients do not respond to neoadjuvant CRT therapy, and the lack of any clinical and pathological factors to guide the initial treatment, novel predictive biomarkers are needed to avoid toxicity and surgery delays. In this context, prior studies have reported the potential usefulness of some gene signatures and microRNA expression profiles to predict neoadjuvant CRT response [14,17]. However, those biomarkers are not well validated for use in daily clinical practice. Here, we show that miR-31 expression levels measured before starting any treatment have the potential ability to predict pathological response, overall survival, and progression-free survival in patients with LARC treated with 5-FU based neoadjuvant CRT.

We decided to evaluate the potential clinical value of miR-31 in predicting CRT response due to the previously-reported oncogenic role of this microRNA in CRC [23]. We found a similar prevalence (around 34%) of miR-31 overexpression to what has been previously reported in the literature (between

10% and 38% depending on the cohort and the stage of the disease) [23,24]. In concordance with our findings, it has been reported that miR-31 suppression increments sensitivity to 5-FU at the initial stage, and also affects invasion and cell migration in HCT-116 cells [22]. Moreover, several studies have reported the potential value of microRNAs to predict neoadjuvant CRT response; however, these works did not evaluate miR-31 expression levels [25,26].

In our study, high miR-31 levels were significantly associated with the lack of pathological response and patients with low miR-31 expression tended to have better response. As indicated above, the pathological response can be predicted with a specificity of 76.3%. Prior works pointed out that the degree of tumor regression might be a very important clinical tool, as it could be used as a prognostic marker; however, the clinical significance of tumor pathological response is still under investigation [7,27]. In our cohort, pathological response is not associated with overall survival (OS) and disease-free survival (DFS). However, we showed that miR-31 deregulation is able to predict pathological response, DFS, and OS. In this way, 78% of the patients with high miR-31 expression were alive at 6 years follow up compared with 96% of those with low miR-31. In addition, 65% of the patients with high miR-31 had a recurrence within the first three years of follow up, compared with 86% of the patients with low miR-31. Those results suggest the strength of miR-31 as a predictive biomarker to guide multidisciplinary treatment in patients with LARC, since cases with high miR-31 had no appreciable clinical benefit from neoadjuvant CRT. Considering these results, high miR-31 patients could benefit from an alternative therapeutic approach, different than 5-FU-based neoadjuvant CRT.

Measuring miR-31 expression levels as a predictive biomarker for neoadjuvant CRT in LARC has the advantage that is easily detected from small amounts of routinely-prepared formalin-fixed paraffin-embedded (FFPE) endoscopic samples by using a RT-PCR. Furthermore, microRNAs have the ability to continue stable, even when subjected to extreme conditions such as very low or high pH levels, boiling, longer storage time, and multiple freeze-thaw cycles [28]. Limitations of our study include the low number of cases included, the lack of validation in an independent larger cohort, and that it was a retrospective study instead of a prospective one. Therefore, the conclusions provided by this study have to be interpreted with caution and the potential clinical usefulness of miR-31 has to be further confirmed in forthcoming studies and controlled randomized clinical trials before a potential inclusion in clinical protocols. However, it is also true that this study is larger than other studies evaluating neoadjuvant CRT treatments, and the treatment regimen was very homogeneous.

Finally, prior works have described the interaction of miR-31 with important tumor suppressor genes, such as the enhancer of zeste homolog 2 (EZH2) [29] and the hypoxia-inducible transcription factor 1α (HIF-1α) [30]. In the same way, in other tumor models, miR-31 represses the regulatory subunit B alpha isoform of the tumor suppressor PP2A (PPP2R2A) [31]. We previously reported that this subunit downregulation is a common event in colorectal cancer patients and its relation with the resistance to 5-FU [32]. According to this and despite there are likely to be multiple explanations for primary or acquired resistance to neoadjuvant CRT—exploring the role and interaction of miR-31 and PPP2R2A to predict neoadjuvant CRT response in LARC patients could be of great interest for future research.

4. Materials and Methods

4.1. Patient Selection

Eighty-one patients diagnosed with LARC and treated with neoadjuvant CRT between the beginning of 2007 and the end of 2012 (6 years) at the University Hospital Fundación Jiménez Díaz, (Madrid, Spain) were retrospectively selected and included in this study. They were followed-up until February 2016.

All patients had a preoperative staging based on transrectal ultrasound (TRUS) and/or magnetic resonance image (MRI) of the pelvis. In addition, a full body computed tomography scan (FBCT) was performed to exclude stage IV disease. The patients were treated with rule-based chemoradiotherapy

regimens based on 5-FU and underwent surgery after one and a half to two months after neoadjuvant CRT completion. Every patient gave written informed consent for tissue storage and analysis at Fundación Jiménez Díaz biobank, Madrid (Spain). Fundación Jiménez Díaz University Hospital, Madrid (Spain) institutional review board approved the study.

4.2. Pathologic Response and Tumor Samples

The Fundación Jimenez Diaz biobank provided the tumor samples. All tissue derived from the surgical resection were classified in concordance with the College of American Pathologist guidelines for invasive carcinomas (TNM, 7th ed.). Two independent pathologists blinded to all patients' clinical data evaluated tumor regression grade according to the modified Ryan classification that categorizes tumors in four levels of response: complete, moderate, minimal, and poor response. Complete response score 0 indicates no viable cancer cells; moderate score 1 indicates single cells or little groups of cancer cells; minimal score 2 indicates residual cancer outgrown by fibrosis; and poor response scores 3 indicates minimal or no tumor kill with extensive residual cancer. Every regression grade was compared with the primary tumor, in concordance with clinical guidelines [33].

4.3. RNA Isolation

We isolated total RNA from formalin-fixed paraffin-embedded (FFPE) tumor biopsies, applying RecoverAll Total Nucleic Acid Isolation kit Ambion (Thermo Fisher Scientific, USA in accordance with manufacturer's instructions. We eluted total RNA and quantified it with a NanoDrop Spectrophotometer (Thermo scientific, Waltham, MA, USA)

4.4. Quantification of MicroRNA Expression Levels

Samples were reverse transcribed using the TaqManHMicroRNA Reverse Transcription Kit (Applied Biosystems Waltham, MA, USA). Working with TaqMan MicroRNA Assays (Applied Biosystems, USA) specific for miR-31 (reference number: 002279) and U6B as internal control, mature miRNAs were quantified by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). TaqMan MicroRNA assays have an ultra-high sensitivity and can detect as few as ten copies of the target transcript in a sample and also have a dynamic range greater than seven logs. Reactions were carried out using an Applied Biosystems 7500 Sequence Detection System. Conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. The ΔC_T method was performed to analyze relative gene expression data.

4.5. Statistical Analysis

We used SPSS v14.0 (SPSS Inc, Chicago, IL, USA) for statistical analyses. We applied the $\chi 2$ test (Fisher exact test) based on bimodal distribution of data to evaluate the correlation between miR-31 overexpression and the clinical and pathological variables. All reported p values are two-sided. We made a receiver operating characteristic (ROC) curve to establish a cutoff for miR-31 expression, calculated its 95% confidence interval, and selected the cutoff point that provides us with the best specificity and sensitivity to differentiate rectal cancer pathological response, in order to assess the potential usefulness of miR-31 as a predictive biomarker. According to this criteria, we considered up-regulation when miR-31 expression levels (ΔC_T) were lower than 0.34. We defined DFS as the time from surgery until recurrence, appearance of a secondary tumor, or death, and OS as the time from the date of diagnosis to the date of last follow-up or death, and applied the Kaplan-Meier method and survival comparisons with the log-rank test and Breslow. Then, we adjusted the Cox proportional hazards model by taking into consideration significant parameters in univariate analysis and considered a p value <0.05 statistically significant. We followed the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guideline [34].

5. Conclusions

In conclusion, our work shows evidence for the potential utility of miR-31 as a predictive biomarker for pathological response and outcome in patients with LARC treated with neoadjuvant CRT—a concept that can be very interesting to better select treatment options in patients with LARC and that can be incorporated into daily clinical practice. However, more studies are needed to better define the biological and clinical significance of miR-31 in rectal cancer. Furthermore, a validation of these results in a larger independent LARC patient cohort is necessary to validate our results.

Acknowledgments: This work was supported by PT13/0010/0012, PI13/02609 and PI15/00934 grants from "Instituto de Salud Carlos III FEDER, Madrid (Spain)".

Author Contributions: Jesús García-Foncillas, Cristina Caramés and Ion Cristobal conceived and designed the experiments; Víctor Moreno, Cristina Caramés, Pablo Minguez and Ion Cristobal performed the experiments and analyzed the data; Juan P. Marín, Paula González-Alonso, Blanca Torrejón, Ana Leon, José I. Martín, María J. Martín, María J. Fernández, Damian García-Olmo, Delia Cortés, Manuel Pedregal, Roberto Hernández, and Federico Rojo contributed reagents/materials/analysis tools and helped to draft the manuscript; Cristina Caramés wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

LARC	Locally advanced rectal cancer
CRT	Chemoradiotherapy
MiR	MicroRNA
MiR-31	MicroRNA-31
FFPE	formalin-fixed paraffin-embedded
5-FU	5-fluorouracil
pCR	pathological complete response; ypT0N0
CRC	colorectal cancer
anti-EGFR	anti-epithelial growth factor receptor
RT-PCR	quantitative real-time polymerase chain reaction
PPP2R2A	regulatory subunit B alpha isoform of the tumor suppressor PP2A
MRI	Magnetic resonance image
TRUS	Transrectal ultrasound
FBCT	full body computed tomography scan
REMARK	Reporting Recommendations for Tumor Marker Prognostic Studies

References

1. Brenner, H.; Kloor, M.; Pox, C.P. Colorectal cancer. *Lancet* **2014**, *383*, 1490–1502. [CrossRef]
2. Sauer, R.; Liersch, T.; Rodel, C. Preoperative *versus* postoperative chemoradiotherapy for locally advanced rectal cancer: Results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. *J. Clin. Oncol.* **2012**, *30*, 1926–1933. [CrossRef] [PubMed]
3. Van Gijn, W.; Marijnen, C.A.; van de Velde, C.J. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12 years follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol.* **2011**, *12*, 575–582. [CrossRef]
4. Chetty, R.; Gill, P.; Govender, D.; Bateman, A.; Chang, H.J.; Deshpande, V.; Driman, D.; Gomez, M.; Greywoode, G.; Jaynes, E.; *et al.* International study group on rectal cancer regression grading: Interobserver variability with commonly used regression grading systems. *Hum. Pathol.* **2012**, *43*, 1917–1923. [CrossRef] [PubMed]
5. MacGregor, T.P.; Maughan, T.S.; Sharma, R.A. Pathological grading of regression following neoadjuvant chemoradiation therapy: The clinical need is now. *J. Clin. Pathol.* **2012**, *65*, 867–871. [CrossRef] [PubMed]
6. Fokas, E.; Liersch, T.; Fietkau, R.; Hohenberger, W.; Beissbarth, T.; Hess, C.; Becker, H.; Ghadimi, M.; Mrak, K.; Merkel, S.; *et al.* Tumor regression grading after preoperative chemoradiotherapy for locally advanced rectal carcinoma revisited: Updated results of the CAO/ARO/AIO-94 trial. *J. Clin. Oncol.* **2014**, *32*, 1554–1562. [CrossRef] [PubMed]

7. Rödel, C.; Martus, P.; Papadoupolos, T.; Füzesi, L.; Klimpfinger, M.; Fietkau, R.; Liersch, T.; Hohenberger, W.; Raab, R.; Sauer, R.; *et al.* Prognostic significance of tumor regression after preoperative chemoradiotherapy for rectal cancer. *J. Clin. Oncol.* **2005**, *23*, 8688–8696. [CrossRef] [PubMed]
8. Gérard, J.P.; Conroy, T.; Bonnetain, F.; Bouché, O.; Chapet, O.; Closon-Dejardin, M.T.; Untereiner, M.; Leduc, B.; Francois, É.; Maurel, J.; *et al.* Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: Results of FFCD 9203. *J. Clin. Oncol.* **2006**, *24*, 4620–4625. [CrossRef] [PubMed]
9. Bosset, J.F.; Collette, L.; Calais, G.; Mineur, L.; Maingon, P.; Radosevic-Jelic, L.; Daban, A.; Bardet, E.; Beny, A.; Ollier, J.C.; *et al.* Chemotherapy with preoperative radiotherapy in rectal cancer. *N. Engl. J. Med.* **2006**, *355*, 1114–1123. [CrossRef] [PubMed]
10. Nagtegaal, I.D.; Gosens, M.J.; Marijnen, C.A.M.; Rutten, H.J.; van de Velde, C.J.; van Krieken, J.H. Combinations of tumor and treatment parameters are more discriminative for prognosis than the present TNM system in rectal cancer. *J. Clin. Oncol.* **2007**, *25*, 1647–1650. [CrossRef] [PubMed]
11. Kim, N.K.; Baik, S.H.; Seong, J.S.; Kim, H.; Roh, J.K.; Lee, K.Y.; Sohn, S.K.; Cho, C.H. Oncologic outcomes after neoadjuvant chemoradiation followed by curative resection with tumor-specific mesorectal excision for fixed locally advanced rectal cancer. *Ann. Surg.* **2006**, *244*, 1024–1030. [CrossRef] [PubMed]
12. Bertolini, F.; Bengala, C.; Losi, L.; Pagano, M.; Iachetta, F.; Dealis, C.; Jovic, G.; Depenni, R.; Zironi, S.; Falchi, A.M.; *et al.* Prognostic and predictive value of baseline and posttreatment molecular marker expression in locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **2007**, *68*, 1455–1461. [CrossRef] [PubMed]
13. Kuremsky, J.G.; Tepper, J.E.; McLeod, H.L. Biomarkers for response to neoadjuvant chemoradiation for rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **2009**, *74*, 673–688. [CrossRef] [PubMed]
14. Casado, E.; Garcia, V.M.; Sanchez, J.J.; Blanco, M.; Maurel, J.; Feliu, J.; Fernandez-Martos, C.; de Castro, J.; Castelo, B.; Belda-Iniesta, C.; *et al.* A combined strategy of SAGE and quantitative PCR provides a 13-gene signature that predicts preoperative chemoradiotherapy response and outcome in rectal cancer. *Clin. Cancer Res.* **2011**, *17*, 4145–4154. [CrossRef] [PubMed]
15. Kong, Y.W.; Ferland-McCollough, D.; Jackson, T.J.; Bushell, M. MicroRNAs in cancer management. *Lancet Oncol.* **2012**, *13*, e249–e258. [CrossRef]
16. Toiyama, Y.; Takahashi, M.; Hur, K.; Nagasaka, T.; Tanaka, K.; Inoue, Y.; Kusunoki, M.; Boland, C.R.; Goel, A. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. *J. Natl. Cancer Inst.* **2013**, *105*, 849–859. [CrossRef] [PubMed]
17. Caramés, C.; Cristóbal, I.; Moreno, V.; del Puerto, L.; Moreno, I.; Rodriguez, M.; Marín, J.P.; Correa, A.V.; Hernández, R.; Zenzola, V.; *et al.* MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. *Int. J. Colorectal Dis.* **2015**, *30*, 899–906. [CrossRef] [PubMed]
18. Bandrés, E.; Cubedo, E.; Agirre, X.; Malumbres, R.; Zárate, R.; Ramirez, N.; Abajo, A.; Navarro, A.; Moreno, I.; Monzó, M.; *et al.* Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Mol. Cancer* **2006**, *5*, 29. [CrossRef] [PubMed]
19. Sun, D.; Yu, F.; Ma, Y.; Zhao, R.; Chen, X.; Zhu, J.; Zhang, C.Y.; Chen, J.; Zhang, J. MicroRNA-31 activates the RAS pathway and functions as an oncogenic MicroRNA in human colorectal cancer by repressing RAS p21 GTPase activating protein 1 (RASA1). *J. Biol. Chem.* **2013**, *288*, 9508–9518. [CrossRef] [PubMed]
20. Slattery, M.L.; Herrick, J.S.; Mullany, L.E.; Valeri, N.; Stevens, J.; Caan, B.J.; Samowitz, W.; Wolff, R.K. An evaluation and replication of miRNAs with disease stage and colorectal cancer-specific mortality. *Int. J. Cancer* **2015**, *137*, 428–438. [CrossRef] [PubMed]
21. Nosho, K.; Igarashi, H.; Nojima, M.; Ito, M.; Maruyama, R.; Yoshii, S.; Naito, T.; Sukawa, Y.; Mikami, M.; Sumioka, W.; *et al.* Association of microRNA-31 with BRAF mutation, colorectal cancer survival and serrated pathway. *Carcinogenesis* **2014**, *35*, 776–783. [CrossRef] [PubMed]
22. Wang, C.J.; Stratmann, J.; Zhou, Z.G.; Sun, X.F. Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. *BMC Cancer* **2010**, *10*, 616. [CrossRef] [PubMed]
23. Manceau, G.; Imbeaud, S.; Thiébaut, R.; Liébaert, F.; Fontaine, K.; Rousseau, F.; Génin, B.; Le Corre, D.; Didelot, A.; Vincent, M.; *et al.* Hsa-miR-31-3p expression is linked to progression-free survival in patients with KRAS wild-type metastatic colorectal cancer treated with anti-EGFR therapy. *Clin. Cancer Res.* **2014**, *20*, 3338–3347. [CrossRef] [PubMed]

DISCUSSION

Study approach discussion

As CRC is a heterogeneous disease, the identification of novel alterations with potential clinical value or molecular targets is necessary for improving the management of patients with CRC. Importantly, the number of approved therapies for mCRC is constantly increasing emphasizing the need for predictive biomarkers.[31] Together with that increasing of approved therapies, oncologic surgery for CRC patients as well as the follow-up protocols are improving, thus leading to an increase in OS rates. However, not all of the patients benefit from current therapies and unfortunately much of the patients relapse after surgery.[54] Despite the better understanding of the molecular heterogeneity of CRC and the current advances in both oncological surgeries and the approval of novel therapies for mCRC, oncologists still suffer a lack of bona fide biomarkers to guide clinical decisions. Although there is abundant functional evidence that some biomarkers can be used to better guide oncologist work, no one has been validated for it use in routine clinical practice with the RAS and BRAF mutations and MMR status exception.[54]

A correct balance between kinases and phosphatases is essential for the correct cellular homeostasis. It is already well known the important role of the gain of function of several kinases for the development of tumors. In fact, several treatments had been investigated for the last decades to block kinases activity.[99] For instance, mCRC therapies such as regorafenib that are based on the inhibition of the kinases activity had been recently approved for mCRC treatment.[80] On the contrary, the role of proteins phosphatases in cancer in less studied. Thus, we focused the present research work in exploring the potential clinical and therapeutic impact of PP2A deregulation in CRC.

After all the works we have performed, we have identified, for the first time, the relevance of the inhibition of PP2A in the pathogenesis of CRC. Furthermore, we identified for the first time some PP2A inhibitory mechanisms that could be used in the future for a better management of CRC patients. Thus, the results of this research work mean another advance in our understanding of genetic and epigenetic alterations that govern CRC pathogenesis. In consequence, our findings should serve to further investigate these alterations as novel valuable clinical biomarkers for prognostic and therapeutic applications in the future.

At the following discussion we are going to go through the clinico-pathological analysis of the different cohorts used, the study methodology strength and limitations as well as to the results analysis and the implications that they can have to the routine clinical practice and for future research projects.

Methodology discussion: study strengths and limitations

Patient selection and samples: As an **initial screening phase** to test PP2A, p-PP2A, SET, CIP2A, PPP2R5E and PPP2R2A status we selected a small cohort of 35 patients that had fresh frozen samples with paired normal and tumor mucosa.

Fresh frozen tissues are ideal for molecular analysis because all their material and nucleotide acids are well preserved. However, such tumor sets are not so abundant and their collection is mainly restricted to tissue banks and research groups.[222]

Once we confirmed the positive preliminary results on that initial cohort, together with the in vitro studies, we went further to the validation phase. In the **validation phase,** we used three independent cohorts where we measured p-PP2A, SET, miR-199b-5p, miR-31 and miR-21, with the objective of further evaluate the potential role of our biomarkers. For those cohorts the samples were from FFPE tissues. We did not measure miR-21 and miR-31 in the initial screening phase because they are already well known oncomiRs in CRC and our main objectives with those two biomarkers was to confirm their expression in LARC and evaluate if there are able to predict 5-FU neoadjuvant CRT. Moreover, miR-199b-5p was not evaluated in the initial phase since its downregulation was later identified as a molecular alteration that led to SET overexpression in around half of mCRC patients with high SET expression levels.

FFPE tissues represent a unique source of archived biological material and are the most widely available source of tissue material for which long-term clinical follow-up data are recorded. Strategies based on FFPE material offer several advantages such as easy handling, long-term cheap storage, and suitability for IHC analyses. The chance to assess gene expression profiling proportioned by the mentioned samples enables the performance of many retrospective and multi-center studies, benefiting the correlation of expression profiles with clinical outcomes. However, the nucleotide acids of FFPE specimens are of much lower quality than RNA obtained from fresh frozen samples. The most remarkable molecular change caused by formalin is the formation of cross-linkages between proteins or between proteins and nucleic acids. Furthermore, several other steps of the paraffin embedding process can affect the quality of the material.[222]

About the cohorts of the validation phase we mainly planned to focus on the two CRC patients groups with worse outcome, mCRC and LARC. Although all of the cohorts where retrospective, the evaluation of our main clinical objectives which were OS and PFS in the mCRC cohort and the pathological response, 0S and PFS in the LARC cohort, are not affected for this issue. Moreover, the evaluation of all the clinical parameters had been carried out by homogenizing all the features thus, trying to guarantee the quality of all the data recorded. It is well know that unfortunately the majority of all the study biomarkers are retrospective with all the advantages and inconvenient that those studies involve.

Finally, we decided to analyze the expression levels of miR-21 and miR-31 in the cohorts of patients with LARC treated with 5-FU based exclusive therapy because that patient cohort was very homogeneous in clinical features and treatment administration. Moreover, as we observed in our preliminary results that PP2A activators drugs had an additive effect to 5-FU alone in CRC cell lines, we wanted to used a clinical model that used 5-FU alone to futher investigated the clinical predictive impact of our biomarkers. As we excluded from that cohort all the patients with other treatments or stages that were not radiotherapy plus 5-FU exclusively we could better identified the role of those two miRs as predictors of response to 5-FU therapies in rectal cancer patients. Limitations of that study cohort include the low number of cases included, the lack of validation in an independent larger cohort, and as mentioned before that it was a retrospective cohort instead of a prospective one. However, it is also true that this study is larger than other studies evaluating neoadjuvant CRT treatment.

MiRs analysis: The development of biomarkers for disease depends in part on their stability and resistance to storage and handling. RNA-based molecular biomarkers has been challenging in the last years due to the effect of RNase present in body fluids affecting their stability. However, endogenous miRs in body fluids and tissues have been shown to remain stable, even when subjected to extreme conditions. Thus, miRs have been proposed as very promising biomarkers in the near future for clinical use.[170]

Therefore, together with the emerging relevant role of miR-21 and miR-31 in CRC[182] and particularly in rectal cancer we decided to measure the impact of these miRs to determine

their possible predictive role to 5-FU in LARC that as mentioned before has not previously been explored to date.

Moreover, measuring miR-31 and miR-21 expression levels as a predictive biomarker for neoadjuvant CRT in LARC has the advantage that is easily detected from small amounts of routinely prepared FFPE endoscopic samples by using a RT-PCR. Furthermore, miRs 31 and 21 as the majority of miRs, are stable even when subjected to extreme conditions such as very low or high pH levels, boiling, longer storage time, and multiple freeze-thaw cycles. In the same way, measurement of miR-199b in FFPE samples of mCRC have also the advantage that is easily detect from small amounts of tissue and the stability with is always a very important consideration when we want to apply the results to the routine clinical practice.

A number of software programs have been developed to predict putative miR targets but all of them produce both false negative and false positive results. For example, use of two target prediction programs, PicTar and TargetScan, returned more than 200 putative miR-31 targets but combination of these with two additional programs, RNAhybrid and miRanda, resulted in only seven common target genes. Experimental validation of the predicted targets is thus essential. A typical first step towards this direction is to overexpress the miR interrogated and to monitor the subsequent changes in the expression levels of the putative target genes. However, more detailed studies, such as luciferase reporter assays are needed to demonstrate that the regulation is indeed direct and not merely a secondary effect.[161 223] In this regard, in our work, we have demonstrated with detailed experimentation, (overexpressing and silencing miR-199b performing in vitro experiments and luciferase assays) that this miR directly controls SET. We did not follow the same procedure with miR-21 and miR-31 but it is within our ongoing goals to further investigate the signaling network affected by PP2A in CRC. Thus, it will be of great interest in future studies to perform in vitro experiments and luciferase assays to fully clarify the role of these miRs modulating PP2A activity in this disease.

Moreover, we did not confirm our results measuring our three miRs in plasma, serum or other body fluids clinical samples. That fact would be really interesting for a nearly future clinical application that could be integrated, if the results are positive, to the further development of liquid biopsies. In addition, it is very important when working with miRs taking into consideration the expression levels normalization. For that reason in our research we applied a well standardized method as explain in materials and methods[220 224]. Finally, for the statistical analysis, considering the importance of all the potential sources of heterogeneity that involve a biomarker study, such as all the treatments, and the multiple clinic-pathological variables, we carried out multivariate analyses including all the significant parameters in the previous univariate analyses.

Results discussion

Role of PP2A inhibition in CRC

PP2A is a tumor suppressor frequently inactivated in several solid and hematological malignancies.[101] Despite there was some little evidence about its relevance contributing to CRC development, its role as an important genetic event in CRC was not known.[119 134 135] We confirmed in the first article of this doctorate thesis that PP2A loss of function is a common event in CRC. In this regard, we showed how PP2A is inactivated in CRC cell lines and patient samples. Moreover, we reported that PP2A restoration using the PP2A activator drug FTY720 reduced CRC cell growth, enhanced apoptosis and showed an addictive effect to 5-FU, irinotecan and oxaliplatin. With those preliminary results we went further to investigate the mechanisms for which PP2A is inhibited in CRC.

PP2A regulatory mechanisms in CRC

It was already well known that cancer cells use several molecular strategies to inhibit PP2A.[225] Between those strategies, are the overexpression of endogenous PP2A inhibitors, such as CIP2A or SET, mutations in some PP2A subunits, miRs deregulation, PP2A-C post-trasncriptional modifications such as hyperphosphorylation or methylation and the downregulation of the PP2A regulatory subunits.[101] However, how PP2A loss of function occurs in CRC was mostly unexplored because, only a few mechanisms such as mutations in several PP2A subunits were described.[226] [227] Nevertheless, the low prevalence reported of those mutations in CRC lead us to investigate other different inhibitory strategies. We identified and describe in articles 1,2,3,4 and 5 of this doctoral thesis several regulatory mechanisms by which PP2A is inhibited in CRC. Below, we are going to discuss them in detail:

Phosphorylation of PP2A catalytic subunit (p-PP2A-C Y307)

The tumor suppressor PP2A has been shown to be functionally inactivated in several types of human cancer through the hyperphosphorylation of its catalytic subunit.[109] Moreover, phosphorylation of tyrosine-307 is responsible for more than 90% of the phosphatase activity of PP2A and it has been shown that this phosphatase is inactive when tyrosine-307 is phosphorylated.[228] However, the relevance of this alteration in CRC remained unknown. That background prompted us to evaluate the role of p-PP2A-C Y307 in CRC. For that, we analyzed its expression in CRC cell lines, performed functional studies and later studied its prevalence in two CRC cohorts. In this regard, we first analyzed the p-PP2A-C status in 7 CRC cell lines, observing that all of them showed PP2A-C hyperphosphorylation. Next, we confirmed on the initial cohort of 35 fresh frozen CRC samples with paired normal mucosa that PP2A-C Y307 is hyperphosphorylated in a subgroup of CRC samples (11 out of 35 samples) and after that, the validation cohort of 250 patients with mCRC demonstrate a high p-PP2A in 17.2% of cases. In addition, the functional studies performed, supported the role of p-PP2A-C Y307 as a strategy by which PP2A is inhibit in CRC.

SET overexpression

Protein SET is a potent PP2A endogenous inhibitor, that is overexpressed in several tumors.[117] To better determine the molecular mechanisms responsible of PP2A inhibition in CRC we decided to assess the expression levels of the PP2A endogenous inhibitor SET observing overexpression of SET in 13 out of 21 cases. Those SET preliminary results indicated that this alteration is a recurrent event in CRC. Moreover, and very interestingly, we observed no alterations in SET in our CRC patients without PP2A inhibition. Going deeply in the study of SET in CRC, we showed in article 4 of the present doctoral thesis that SET is overexpressed in CRC cell lines and plays an oncogenic role promoting cell proliferation, colonosphere formation and impairing PP2A antitumor activities. Moreover, to study the functional importance of SET deregulation, we silenced SET with two different specific siRNAs in six CRC cell lines and observed that in all cases SET silencing induced a decrease in the cell growth and colonosphere formation ability in both number and size of the spheres formed. Similarly, SET overexpression inhibits PP2A then increasing cell growth and decreasing apoptosis. All together, these results indicate that SET overexpression is an alteration that plays an oncogenic role in CRC through the inhibition of PP2A activity.

MiR-199b-5p downregulation

Once we demonstrated that SET overexpression is a contributing mechanism of PP2A inhibition with an important oncogenic role in CRC we also wanted to explore how SET deregulation occurs in CRC because this fact remains fully unknown.

We then evaluated in the literature molecular causes that could lead to SET overexpression in CRC. MiR-199b-5p downregulation is reported to be a cause of SET overexpression in some malignancies.[139] However, this fact in CRC was not studied. Moreover, there were no papers in the medical literature exploring miR-199b-5p role in CRC. Thus, we analyze whether an altered expression of miR-199b-5p could be involved in CRC development and in SET deregulation in this maligancy and therefore, affect PP2A activity. With that objective, we first quantified miR-199b-5p in 5 CRC cell lines previously reported to have SET overexpression, observing that miR-199b-5p was downregulated in 4 out of 5 cases. Moreover, we quantified miR-199b-5p expression levels as well as SET levels in a cohort of mCRC patients observing an inverse correlation. Those observations, published in article 5 of this doctoral thesis, were the first positive results to suggest the role of miR-199b-5p in SET-mediated PP2A inhibition in CRC. This issue was further supported by the decrease in SET levels together with PP2A activation observed after miR-199b-5p overexpression in CRC cell lines. Of note, the transfection with anti-miR-199b-5p only promoted a slight reduction in PP2A activity probably due to the low basal miR-199b-5p expression together with the SET overexpression status in both SW480 and HT-29 cells. These findings are in concordance with the fact that anti-miR-199b-5p only induced a discrete increase of cell viability whereas miR-199b-5p overexpression led to a marked reduction of cell growth. Furthermore, the antitumor effects of miR-199b-5p on cell growth is probably due to its role as negative SET regulator since the co-expression of miR-199b-5p together with SET almost totally restored proliferation of CRC cells. Finally, we also performed a luciferase assay to validate the role of miR-199b as a negative SET regulator in CRC.

All those results confirmed for the first time that miR-199b-5p emerges as a novel tumor suppressor miR in CRC and that its downregulation is a common alteration that contributes to PP2A inhibition in this disease. Interestingly, at the same time that we publish our paper related to the role of miR-199b-5p as a tumor suppressor miR in CRC another group supported our findings with a publication with similar results but proposing another target to miR-199b-5p that was SIRT1. Similarly to our results, the authors showed how the transfection of miR-199b-5p suppressed CRC cell invasion and migration both in vitro and in vivo.[229]

Downregulation of regulatory PP2A subunits: PPP2R2A and PPP2R5E

Together with the overexpression of endogenous PP2A inhibitors and cancer-associated mutations affecting structural PP2A subunits, downregulation of regulatory PP2A subunits has been described as a mechanism to inactivate PP2A in cancer cells.[101]

Alterations affecting the activity of PPP2R2A have been associated with cancer progression.[126] For instance, somatic deletions in PPP2R2A had been related to prostate cancer development[230] and alterations affecting PPPR2A facilitated lung tumors development.[212] In the same way, alterations affecting the activity of PPP2R5E had been associated with low PP2A activity in cancers such as soft tissue sarcoma[231] and AML.[125] However, how the alterations in those subunits contribute to PP2A inactivation in CRC had not previously been studied.

Thus, we analyzed PPP2R2A and PPP2R5E in CRC samples fresh frozen samples and paired normal mucosa, observing downregulation of these subunits in, respectively, nine and three out of the 21 CRC cases. Those alterations could contribute to explain some of the differences observed in the PP2A activity levels of the patients with CRC. However, PP2A constitutes a

large family of serine/threonine phosphatases, and in this work, we have only tested these two PP2A regulatory subunits by its previously reported relevance in human cancer. Further research of the role of those PP2A regulatory subunits in CRC would be of great interest.

Oncogenic miR-31 and miR-21 overexpression

MiR-31 plays an important role in CRC development and is one of the miRs better studied in CRC.[161] Moreover, miR-31 is overexpressed in lung cancers and it acts as an oncogenic miR by targeting the PP2A regulatory subunit PPP2R2A,[212] whose downregulation was previously identified in the first paper of this thesis as a contributing PP2A inhibitory alteration in CRC. At our research work, we showed that PP2A activation status modulates the response to 5-FU in CRC. In this line of thinking, we also observed a role of miR-31 and miR-21 in rectal cancer determining poor prognosis and 5-FU therapy resistance. A potential explanation could be that miR-31 is controlling 5-FU sensitivity via PPP2R2A regulation. This fact would be of great interest to study and confirm in future studies.

On the other hand, miR-21 is a well-understood oncogenic miR in CRC and also one of the most studied.[161] It has been reported that PP2A holoenzymes containing PPP2R2C negatively regulates the MAPK pathway through inhibition of c-SRC[101], which increases miR-21 expression.[232] As previously indicated, c-SRC phosphorylates and inhibits PP2A [233] [213] and the enhancement of miR-21 expression could serve to reinforce its role as PP2A inhibitor since miR-21 targets PPARα, which upregulates and activates PP2A. Therefore, miR-21 would be contributing to inhibit PP2A via PPARα downregulation.[234] Moreover, miR-21 could be affecting through PTEN targeting the activation status of ERK and AKT, which represent two key PP2A targets responsible of at least part of the PP2A tumor suppressor roles in human cancer.[99] [101]

Finally, miR-21 expression is regulated by the STAT3 and NFKB transcription factors, which are both constitutively activated in a variety of cancers and play critical roles in the regulation of cell proliferation, invasion, apoptosis, and tumorigenesis. It was, also being reported that some PP2A subunits can have a role in the regulation of NFkB. Those molecular relations in CRC should be of great interest to evaluate in future studies.[217]

Potential novel biomarkers based on PP2A inhibition for CRC

We discuss below how the new molecular alterations in CRC that we identified can emerge as novel biomarkers with therapeutic applications for CRC.

PP2A catalytic subunit hyperphosphorylation as a prognostic biomarker

It had been previously reported that p-PP2A is a recurrent event and confers a worse outcome in some hematological malignancies.[125] However, the role of this alteration in CRC had not been explored. Of importance, we report in the manuscipt 2 of this doctoral thesis that PP2A hyperphosphorylation represents a recurrent molecular event in mCRC associated with worse ECOG performance status and the presence of synchronous metastasis. Importantly, this alteration determines a markedly shorter OS and PFS, and patients with high p-PP2a levels have a median PFS of 3.8 months vs 13.3 months for the patients with low p-PP2A. Moreover, the subgroup of patients with high p-PP2A expression has a median OS of 6 months vs 26.2 months for the patients with low p-PP2A. This poor outcome was especially notable in the subgroup of patients younger than 70 years and the prognostic impact was similar in the KRAS wild type and mutated subgroups. In summary, our results confirm the clinical relevance of p-PP2A as a prognostic biomarker in mCRC. In addition, the fact that the

prognostic impact of p-PP2A showed higher significance in those patients younger than 70 years is very interesting since this subgroup includes cases with more options from a therapeutic perspective that could benefit from the treatment with PP2A activating drugs.

SET overexpression as a prognostic and FOLFOX therapy predictive biomarker

Deregulated expression of SET confers worse outcome in patients with some hematological malignancies.[125] However, its potential functional and clinical relevance in CRC had not been explored at the beginning of this doctorate. Interestingly, our findings showed that SET modulates in vitro sensitivity of CRC cells to both 5-FU and oxaliplatin treatments in CRC cell lines. We also analyzed the potential significance of SET overexpression in a series of 242 cases with mCRC. This subgroup of CRC patients represents the highest therapeutic challenge and for whom it is necessary to develop alternative therapeutic strategies that improve their current still very poor outcome. Importantly, we observed that SET overexpression is a recurrent event (24.8%) that predicts an adverse OS and PFS in patients with mCRC. In addition, we analyzed SET in a series of 145 patients with CRC without metastatic disease, observing a significant lower prevalence of SET overexpression in this cohort than in the metastatic cohort (13.8% vs. 24.8%). In concordance with our in vitro results, the subgroup of FOLFOX-treated patients and high SET expression levels also showed a significant worse PFS and OS.

Thus, our findings suggest that SET overexpression could discriminate a subgroup of patients with worse outcome who might not benefit of oxaliplatin therapies and for whom alternative therapies based in pharmacological PP2A reactivation should be considered.

MicroRNA 199b-5p downregulation as a prognostic and FOLFOX predictive biomarker

There is some data in the literature describing that miR-199b-5p plays a tumor suppressor role and its downregulation in some human cancer as a prognostic biomarker associated with a worse outcome.[195 196 204] Furthermore, miR-199b-5p has been reported to be involved in acquired resistance to different antitumor therapies in human cancer such as imatinib in CML[200], cisplatin in ovarian cancer[201] or trastuzumab in breast cancer.[204] However, its status and importance in CRC remained to be investigated.

Therefore and considering that miR-199b-5p downregulation seems to be a molecular cause of SET overexpression in a subgroup of mCRC patients, we hypothesized that this alteration could have clinical impact in mCRC. As we reported in the article 5 of this doctoral thesis, miR-199b-5p downregulation determined poor outcome and a poor clinical benefit of 5-FU and oxaliplatin therapies, predicting lack of response in those cases treated with FOLFOX-based chemotherapy. Patients with high miR-199b-5p expression have a medium PFS and OS longer than patients with low expression. Moreover, of the total 39 patients studied that were treated with oxaliplatin, eight had low expression of miR-199b-5p and all of then had a progression. On the contrary, 31 out of 39 had high expression of miR-199b-5p and nearly half of then didn't show a progression suggesting the emergin role of miR-199b-5p as a predictive biomarker of response to oxaliplatin therapies.

Our results are supported by a recently publication in which the authors explored the role of miR-199b-5p in CRC progression and metastasis. The authors determined that low miR-199b-5p expression predicts poor prognosis in a series of 60 CRC patients and miR-199b-5p expression levels determine response to oxaliplatin.[198]

In addition to SET, miR-199b-5p has been reported to regulate other important targets such as HEIS1[202], HIF1α[197] or HER2[204] in medulloblastoma, hepatocellular carcinoma and breast

cancers respectively. In our cohort, among those mCRC patients without SET overexpression, we observed miR-199b-5p downregulated in 7 out of 65 cases. Of importance, we observed that miR-199b-5p downregulation determined substantially shorter OS in these patients. Although significance was not achieved probably because the low number of cases studied, these observations indicate a potential SET-independent prognostic value for miR-199b-5p that needs to be further confirmed in future studies.

MicroRNA 21 and 31 overexpression as potential predictive biomarkers in LARC

Neoadjuvant chemoradiotherapy is the standard treatment for stage II/III rectal cancer.[10] [54] Compared with postoperative therapy, it improved local control and tolerability.[49] [52] However, 5-FU-based CRT, which is the gold standard for those patients, produces a complete pathologic response in only 8% to 14% of the patients, and distant metastases develop in one-third of the cases after 5 years. Furthermore, patients who do not respond to preoperative CRT are exposed to unnecessary toxicities and resection of their primary tumor is delayed. Thus, the ability to predict treatment response and outcome before CRT based on 5-FU is of key importance to clinicians.[235]

Based on the ability of miR-31 and miR-21 to affect PP2A signaling and taking into account our previous data indicating that PP2A activation status modulates sensitivity of CRC cells to 5-FU, together with other data in the literature indicating the potential role of these miRs in rectal cancer and 5-FU resistance, we aimed to evaluate the potential clinical value of those two miRs determining neoadjuvant CRT response in LARC patients.

In our study, high miR-31 levels were found in 34% of the patients and were significantly associated with the lack of pathological response. Moreover, patients with low miR-31 expression tended to have better response. We showed in our series that the pathological response could be predicted with a specificity of 76.3% based on MiR-31 expression. Furthermore, 78% of the patients with high miR-31 expression were alive at 6 years follow up compared with 96% of those with low miR-31. In addition, 65% of the patients with high miR-31 had a recurrence within the first three years of follow up, compared with 86% of the patients with low miR-31.

On the other hand, high miR-21 levels were found in 77.6% of the patients. Similarly, we also reported that miR-21 is able to predict pathologic response in samples of patients with LARC before neoadjuvant CRT. Thus, high levels of miR-21 were significantly associated with the lack of pathological response. We showed in our series that based on miR-21 expression levels pathological response could be predicted with a sensitivity of 86.6%.

Altogether, our results suggest the strength of miR-31 and miR-21 as predictive biomarkers to guide multidisciplinary treatment in patients with LARC, since cases with low miR-21 expression are likely to have a complete pathological response but not those cases with miR-21 high and patients with miR-31 high had no clinical benefit from neoadjuvant CRT. Considering these results, they could be of great interest helping to select patients who are not going to positively respond to standard therapy to receive an alternative therapeutic approach, different than 5-FU-based neoadjuvant CRT.

PP2A as a novel therapeutic target in CRC

FTY720 (Fingolimod, Gilenya) is a structural analogue of sphingosine developed from the fungal metabolite myriocin.[236] The potential anticancer mechanism of FTY720 is through the inhibition of the proto-oncogene sphingosine kinase 1. In addition, FTY720 anticancer

properties may be attributable to actions on several other molecular targets such as S1P receptors, autotaxin and PP2A.[237]

FTY720 has demonstrated anticancer properties. In vitro and in vivo studies demonstrated the growth arrest and apoptosis- inducing ability of FTY720 in several normal and cancer cells including: lymphocytes,[238] microglia,[239] prostate cancer,[240] breast cancer,[241] several forms of leukaemia and lymphoma,[133] [242] lung cancer,[243] liver cancer,[244] pancreatic cancer,[245] and others.[237] However, the effect of this drug in CRC was almost unexplored. The only report was the study from Nagaoka and colleagues where they suggest the effect of FTY720 phosphorylation in CRC cell lines.[246]

In our research work, we demonstrate in vitro using a panel of CRC cell lines that the PP2A activator FTY720 induced PP2A activation leading to cell growth inhibition, impaired clonogenic potential, caspase-dependent apoptosis, and changes in the activation status of downstream PP2A targets such as AKT and ERK1/2. Importantly, FTY720 treatment shows an additive effect with the chemotherapy reagents 5-FU, SN-38, and LOHP, suggesting that treatment with PP2A activators could be a novel therapeutic option in CRC in combination with standard chemotherapy.

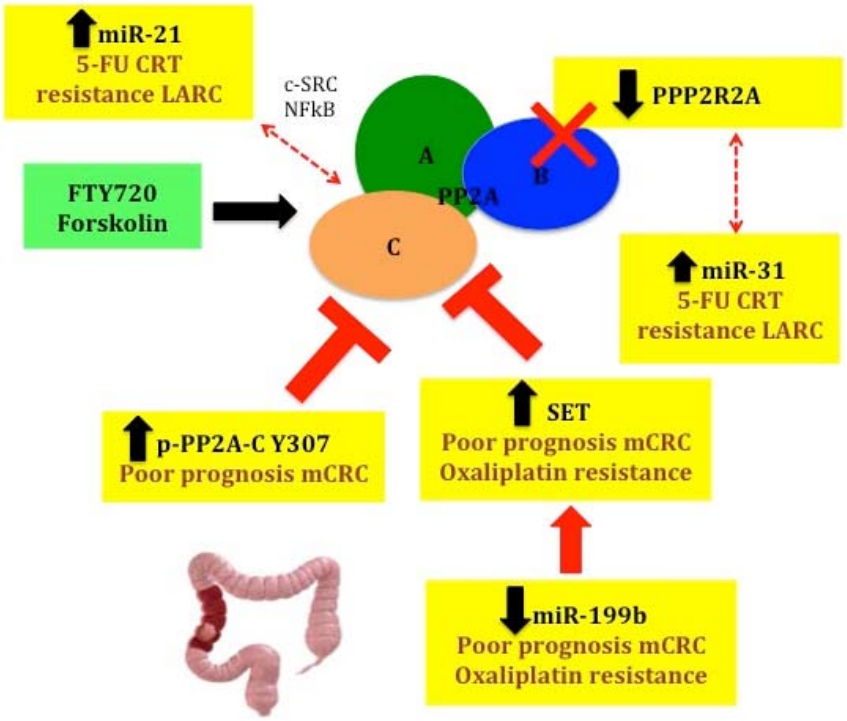
In addition, in the third article we report that forskolin treatment (a drug that has previously been reported to activate PP2A in association with its dephosphorylation[133]), dephosphorylated and increased PP2A activity, impairing proliferation and colonosphere formation, and inducing activation of caspase 3/7 and changes in the phosphorylation status of AKT and ERK. Interestingly, we also reported that forskolin showed additive effects with either 5-FU or oxaliplatin treatments, suggesting the use of PP2A activators such as forskolin for future trials in combination with these standard chemotherapy drugs. Of note, we reported in articles 2 and 3, that PP2A-C is hyperphosphorylated in a subgroup of CRC patient. Together with the in vitro results, indicates the potential therapeutic use of PP2A-C dephosphorylating drugs in those patients, alone or in combination with standard chemotherapy agents.

Global results discussion

PP2A is a tumor suppressor that has the ability to form a high number of different complexes with an also high number of potential alterations that could contribute to PP2A inhibition affecting any of the PP2A subunits.[101] In fact, the cancerous cell shows a wide variety of molecular strategies to inhibit PP2A, including hyperphosphorylation of its catalytic subunit, downregulation affecting any of the scaffold or regulatory subunits, and the overexpression of endogenous PP2A inhibitors such as SET or CIP2A, but also others such miRs deregulation.[99] [126] However, PP2A status, role, regulation and clinical significance in CRC had not been explored.

This research work has allowed to clarify the particular importance of PP2A inhibition in CRC, some mechanisms of PP2A inhibition in this disease and identified other potential mechanisms that would be very interesting to further investigate with more detail. Moreover, this research work has permitted to identify these alterations as potential important tools for use in clinical practice as they can define subgroups of patients with poor prognosis or bad response to current therapies. For those patients, alternative therapies would be relevant. The Figure 19 summarizes the results of this doctorate thesis.

Figure 19. Global results illustration of the doctorate thesis



First of all, we have demonstrated that PP2A inhibition is a key molecular mechanisms for CRC pathogenesis in some patients because it increases clonogenic potential and cell growth, decrease apoptosis and its expression is low in a significant amount of patients with CRC compared with the normal colon tissue. Based on that and taking into consideration some strategies that cancer cell use to inhibit PP2A in other types of cancer[101] [117] we aimed to investigate the molecular PP2A inhibitory mechanisms in CRC. Thus, we have demonstrated different alterations by which PP2A activity is decrease in CRC such as SET overexpression and the hyperphosphorylation of the PP2A catalytic subunit. Moreover, we have identified the deregulation of PPP2R2A which is a target of miR-31[212] as a potential contributing to PP2A inhibition and miR-199b-5p as a regulatory mechanism of SET.

Our results about the importance of the inhibition of PP2A in CRC are endorsed and confirmed since the number of publications related to PP2A and CRC since 2014 has exponentially increase from almost none report to almost forty. As some examples, it has recently been published that the depletion of ATM inhibits colon cancer proliferation and migration via B56γ2-mediated Chk1/p53/CD44 cascades.[247] Also it has been reported that the mTOR and PP2A pathways regulate PHD2 phosphorylation to fine-tune HIF1α levels and CRC cell survival under hypoxia.[248] Another important report explains how the survival mediated by PP2A-STAT3 determines tumor initiation and metastasis in CRC stem cells.[249]

Despite we have not investigated all the alterations described as PP2A inhibition mechanisms contributing as potential biomarker in CRC; we have explored the role of some of them. In this regard, considering our results we propose high p-PP2A, SET overexpression and miR-199b downregulation as emerging prognostic biomarkers for mCRC. Moreover, SET overexpression and miR-199b would also serve as predictors of resistance to oxaliplatin-based therapies in mCRC and miR-21 and, miR-31 overexpression as predictor of response to 5-FU based neoadjuvant CRT in LARC. Concerning this, our results related to miR-21 overexpresion

in LARC were confirmed by Lopes-Ramos et al soon after our report.[250]

Finally, as PP2A activator drugs seems to work in CRC patients cells lines through restoration of PP2A activityand PP2A activators drugs increases the effect of current cytotoxic therapies such as oxaliplatin and 5-FU, we have identified three subgroups of mCRC patients for whom those PP2A activators therapies could represent a potential therapeutic alternative.

Clinical applicability of the results obtained and future perspectives

We confirmed that SET and miR-199b-5p deregulation are two recurrent alterations that contribute to inactivate PP2A and enhance tumor malignant properties of CRC cells.[4] [5] Interestingly, together with their prognostic value, SET and miR-199b-5p are predictive of resistance to oxaliplatin-based chemotherapy, which could have an important relevance in the current clinical practice. Thus, SET and/or microRNA 199b-5p could differentiate a subgroup of patients who should not receive oxaliplatin based therapies if those results are confirmed in a large and prospective clinical trial. Also, SET overexpression and miR-199b-5p deregulation defines a subgroup of patients that could benefit by the future incorporation of PP2A-activating drugs, alone or in combination with standard chemotherapy.

In the same way p-PP2A is a recurrent alteration in a subgroup of mCRC that confer a worse prognosis.[2] Thus, knowing this alteration can contribute to a better management in mCRC. If those result are confirm, probably, those patients should went into protocols were more intensive therapies are apply.

Also, when facing with a patient with a recently diagnosis of LARC, taking into consideration the expression levels of miR-21 and miR-31 should optimize its management. If those miRs are overexpressed an alternative treatment for the standard one (5-FU based neoadjuvant CRT) should be considered.[6] [7]

Ideally, all miRs and molecular alterations that consistently show a predictive or prognostic value in retrospective studies, should be considered for prospective validation.[31] Therefore, it would be of great interest that future clinical trials focus on the discovery of novel biomarkers in different tissue types (plasma, DNA, tumor, primary tissue). Furthermore, since it is becoming clear that a single biomarker may not be sufficient to predict the complex phenotype of response to therapies, it would be very interesting that the validation studies integrate the individual effects of these markers and combine them into a general predictive score.

To finish, this research work ends opening new fields of research that could be the PhD projects of other physicians of our research group.

First, it would be of great interest to further investigate alternative PP2A inhibitory mechanisms based on miR deregulation such as the influence of miR-375 or miR-200c in CRC through PP2A deregulation. Also, it would be very important to deeply investigate the role of miR-21 and miR-31 in modulating PP2A activation status in CRC trough c-SRC and/or NFkB and PPP2R2A deregulation, respectively.

Next, it would be of high clinical relevance to investigate the correlation of our potential biomarkers found deregulated in CRC tissue samples in body fluids such as blood. In this regard, it is already well known the importance of the “liquid biopsy” for the management of cancer patients. Of note, liquid biopsies allow a better monitoring of the response to therapies, assess minimal residual disease, uncover the emergence of drug resistance, allow a continuous therapeutic assessment, reduce the needs of tumor tissue biopsies (and thus invasive procedures) and optimize samples and costs.

Moreover, to investigate the potential therapeutic ussefulness of PP2A activator drugs such as forskolin and FTY720 in patients with PP2A inhibition due to SET overexpression, miR-199b-5p downregulation, and high p-PP2A-C expression levels, would be of great interest. For that, it will be necessary to carry out a phase I clinical trial selecting patients harboring those molecular alterations and offering the PP2A activator drugs alone or in combination with current cytotoxic therapies.

CONCLUSIONS

1. PP2A plays a tumor suppressor role and its inhibition is a common event in CRC.

2. SET overexpression and PP2A hyperphosphorylation represent key PP2A inhibitory alterations in CRC. Moreover, miR-199b-5p dowregulation importantly contributes to SET overexpression in CRC, and downregulation of the regulatory PP2A subunits PPP2R2A and PPP2R5E are significantly present in CRC patients probably contributing to PP2A loss of function.

3. PP2A hyperphosphorylation, SET overexpression and miR-199b-5p dowregulation are potential biomarkers in mCRC. In fact, they independently confer worse outcome to patients with mCRC, and downregulation of miR-199b-5p and SET overexpression also determine the response to oxalipatin in patients with mCRC.

4. Overexpression of miR-31 and miR-21, two closely PP2A-related miRs, determine pathological response to 5-FU neoadjuvant CRT in LARC patients.

5. The PP2A activators drugs forskolin and FTY720 restore PP2A activity thereby showing promising antitumor effects in CRC cell lines. This fact together with the additive effect that these drugs have shown with currently used cytotoxic chemotherapy regimens strongly suggest further investigation about the potential therapeutic benefit derived from the clinical use of PP2A activators in CRC patients harboring PP2A inhibitory alterations.

CONCLUSIONES

1. PP2A tiene un papel como supresor tumoral en cancer colorrectal y su inhibición es una alteración molecular frecuente en esta enfermedad.

2. La sobreexpresión de SET y la hiperfosforilación de la subunidad catalítica de PP2A son alteraciones que juegan un papel clave en la inhibición de PP2A en cáncer colorrectal. Además la baja expresión del microRNA-199b contribuye a la sobreexpresión de SET, y la menor expresión de las subunidades reguladoras de PP2A, PPP2R2A y PPP2R5E, están presentes de forma significativa en cáncer colorectal contribuyendo a su pérdida de función.

3. La hiperfosforilación de PP2A, la sobreexpresión de SET y la baja expresión del microRNA-199b-5p son potenciales biomarcadores en cáncer colorrectal. Así, estas tres alteraciones determinan de forma independiente un mal pronóstico para los pacientes con cáncer colorectal metastasico, y la baja expresión del microRNA 199b y la sobreexpresión de SET confieren además falta de respuesta al tratamiento con oxaliplatino.

4. La sobreexpresión de los microRNAs-31 y 21, dos microRNAS estrechamente relacionados con PP2A, es capaz de determinar la respuesta al tratamiento con quimioradioterapia neoadyuvante basado en 5-fluorouracilo en pacientes con cáncer

de recto localmente avanzado.

5. Los fármacos activadores de PP2A FTY720 y forskolina han inducido prometedores efectos antitumorales en líneas celulares de cáncer colorrectal a través del restablacimiento de la actividad de PP2A. Este hecho, junto con el efecto aditivo que estos compuestos han mostrado en combinación con fármacos quimioterápicos estándar, abre una nueva línea de investigación según la cual sería interesante estudiar el potencial beneficio terapéutico derivado del uso clínico de estos fármacos activadores de PP2A en pacientes que presenten alteraciones inhibidoras de PP2A.

ANNEXES

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BIBLIOGRAPHY

1. Cristobal I, Manso R, Rincon R, Caramés C et al. PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. Mol Cancer Ther 2014;13(4)938-47.
2. Cristóbal I, Manso R, Rincón R, Caramés C et al. Phosphorylated protein phosphatase 2A determines poor outcome in patients with metastatic colorectal cancer. Br J Cancer 2014;111(4):756–62.
3. Cristóbal I, Rincón R, Manso R, Caramés C et al. Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation. Biochim Biophys Acta 2014;1842(9):1823–9.
4. Cristobal I, Rincon R, Manso R, Caramés C et al. Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer. Clin Cancer Res 2015;21(2):347–56.
5. Cristóbal I, Caramés C, Rincón R, et al. Downregulation of microRNA-199b predicts unfavorable prognosis and emerges as a novel therapeutic target which contributes to PP2A inhibition in metastatic colorectal cancer. Oncotarget 2016; In press
6. Caramés C, Cristobal I, Moreno V, et al. MicroRNA-31 Emerges as a Predictive Biomarker of Pathological Response and Outcome in Locally Advanced Rectal Cancer. Int J Mol Sci 2016;17(6):878.
7. Caramés C, Cristóbal I, Moreno V, et al. MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. Int J Colorectal Dis 2015;30(7):899-906
8. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global Cancer Statistics, 2012. CA Cancer J Clin 2015;65(2):87-108
9. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. Cancer Epidemiol Biomarkers Prev 2009;18(6):1688–94.
10. Brenner H, Kloor M, Pox CP. Colorectal cancer. Lancet 2014;383(9927):1490–502.
11. Siegel R, Desantis C, Virgo K, et al. Cancer Treatment and Survivorship Statistics, 2012. CA Cancer J Clin 2012;62(4):220–41.
12. Jess T, Rungoe C, Peyrin-Biroulet L. Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies. Clin Gastroenterol Hepatol 2012;10(6):639–45.
13. Liang PS, Chen T-Y, Giovannucci E. Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. Int J Cancer 2009;124(10):2406–15.
14. Fedirko V, Tramacere I, Bagnardi V, et al. Alcohol drinking and colorectal cancer risk: An overall and dose-Response meta-analysis of published studies. Ann Oncol 2011;22(9):1958–72.
15. Chan DSM, Lau R, Aune D, et al. Red and processed meat and colorectal cancer incidence: Meta-analysis of prospective studies. PLoS One 2011;6(6):e20456
16. Ma Y, Yang Y, Wang F, et al. Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies. PLoS One 2013;8(1):e53916

17. Jiang Y, Ben Q, Shen H, Lu W, Zhang Y, Zhu J. Diabetes mellitus and incidence and mortality of colorectal cancer: A systematic review and meta-analysis of cohort studies. Eur J Epidemiol 2011;26(11):863–76.

18. Kostic AD, Gevers D, Pedamallu CS, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res 2012;22(2)292–8.

19. Inoue I, Kato J, Tamai H, et al. Helicobacter pylori-related chronic gastritis as a risk factor for colonic neoplasms. World J Gastroenterol 2014;20(6):1485–92.

20. Boyle T, Keegel T, Bull F, Heyworth J, Fritschi L. Physical activity and risks of proximal and distal colon cancers: A systematic review and meta-analysis. J Natl Cancer Inst 2012;104(20):1548–61.

21. Lin KJ, Cheung WY, Lai JYC, Giovannucci EL. The effect of estrogen vs. combined estrogen-progestogen therapy on the risk of colorectal cancer. Int J Cancer 2012;130(2):419–30.

22. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: A systematic comparison of evidence from observational studies versus randomised trials. Lancet Oncol 2012;13(5):518–27.

23. Elmunzer BJ, Hayward RA, Schoenfeld PS, Saini SD, Deshpande A, Waljee AK. Effect of Flexible Sigmoidoscopy-Based Screening on Incidence and Mortality of Colorectal Cancer: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. PLoS Med 2012;9(12):1–9.

24. Aune D, Chan DSM, Lau R, et al. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. Bmj 2011;343(1):d6617

25. Aune D, Lau R, Chan DSM, et al. Dairy products and colorectal cancer risk: A systematic review and meta-analysis of cohort studies. Ann Oncol 2012;23(1):37–45.

26. Wu S, Feng B, Li K, et al. Fish consumption and colorectal cancer risk in humans: a systematic review and meta-analysis. Am J Med 2012;125(6):551–9.

27. Von Delius S. Statins and colorectal cancer prevention. Z Gastroenterol 2006;44(2):543–4.

28. Ma Y, Zhang P, Wang F, Yang J, Liu Z, Qin H. Association between vitamin D and risk of colorectal cancer: A systematic review of prospective studies. J Clin Oncol 2011;29(28):3775–82.

29. De Sousa E Melo F, Wang X, Jansen M, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat Med 2013;19(5):614–8.

30. Sadanandam a, Lyssiotis C a, Homicsko K, et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat Med 2013;19(5):619–25.

31. Okugawa Y, Grady WM, Goel A. Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. Gastroenterology 2015;149(5):1204–1225e.

32. Carethers JM, Jung BH. Genetics and Genetic Biomarkers in Sporadic Colorectal Cancer. Gastroenterology 2015;149(5):1177–90.

33. Fearon EF, Vogelstein B. A genetic model for Colorectal Tumorigenesis. Cell 1990;61(5):759–67.

34. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz L a, Kinzler KW. Cancer Genome Lanscapes. Science 2013;339(6127):1546–58.

35. Tomasetti C, Marchionni L, Nowak MA, Parmigiani G, Vogelstein B. Only three driver gene mutations are required for the development of lung and colorectal cancers. Proc Natl Acad Sci U S A 2015;112(1):118–23.

36. Network TCGA, Muzny DM, Bainbridge MN, et al. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487(7407):330–7.

37. Xu L, Zhang Y, Wang H, Zhang G, Ding Y, Zhao L. Tumor suppressor miR-1 restrains epithelial-mesenchymal transition and metastasis of colorectal carcinoma via the MAPK and PI3K/AKT pathway. J Transl Med 2014;12:244.

38. Garraway LA, Lander ES. Lessons from the cancer genome. Cell 2013;153(1):17–37.

39. Hamilton W, Round A, Sharp D, Peters TJ. Clinical features of colorectal cancer before diagnosis: a population-based case-control study. Br J Cancer 2005;93(4):399–405.

40. Al-Sukhni E, Milot L, Fruitman M, et al. Diagnostic Accuracy of MRI for Assessment of T Category, Lymph Node Metastases, and Circumferential Resection Margin Involvement in Patients with Rectal Cancer: A Systematic Review and Meta-analysis. Ann Surg Oncol 2012;19(7):2212–23.

41. Leufkens AM, van den Bosch M a a J, van Leeuwen MS, Siersema PD. Diagnostic accuracy of computed tomography for colon cancer staging: a systematic review. Scand J Gastroenterol 2011;46(78):887–94.

42. Niekel, M C, Bipat S, Stoker J. Diagnostic Imaging of Colorectal Liver Metastases with CT, MR Imaging, FDG PET, and/or FDG PET/CT: A Meta-Analysis of Prospective Studies Including Patients Who Have Not Previously Undergone Treatment. Radiology 2010;257(3):674–84.

43. Bass AJ, Thorsson V, Shmulevich I, et al. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014;513(7517):202–9.

44. Gill S, Loprinzi CL, Sargent DJ, et al. Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: Who benefits and by how much? J Clin Oncol 2004;22(10):1797–806.

45. Peeters M, Price TJ, Cervantes A, et al. Randomized Phase III Study of Panitumumab With Fluorouracil, Leucovorin, and Irinotecan (FOLFIRI) Compared With FOLFIRI Alone As Second-Line Treatment in Patients With Metastatic Colorectal Cancer. J. Clin. Oncol. 2010;28(31):4706–13.

46. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: Updated results of NSABP C-07 trial, including survival and subset analyses. J Clin Oncol 2011;29(28):3768–74.

47. Van Cutsem E, Labianca R, Bodoky G, et al. Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. J Clin Oncol 2009;27(19):3117–25.

48. QUASAR Collaborative Group. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet 2007;370(9604):2020–9.

49. Sauer R, Becker H, Hohenberger W, et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N Engl J Med 2004;351(17):1731–40.

50. Mamon HJ. Long-term follow-up of a paradigm-changing study: The paradigm still

holds. J Clin Oncol 2012;30(16):1901–3.

51. Van Gijn W, Marijnen CAM, Nagtegaal ID, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. Lancet Oncol 2011;12(6):575–82.

52. Hofheinz RD, Wenz F, Post S, et al. Chemoradiotherapy with capecitabine versus fluorouracil for locally advanced rectal cancer: A randomised, multicentre, non-inferiority, phase 3 trial. Lancet Oncol 2012;13(6):579–88.

53. Aschele C, Cionini L, Lonardi S, et al. Primary tumor response to preoperative chemoradiation with or without oxaliplatin in locally advanced rectal cancer: Pathologic results of the STAR-01 randomized phase III trial. J Clin Oncol 2011;29(20):2773–80.

54. Van Cutsem E, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol 2016;27(8):1386-422

55. Schmoll HJ, Van cutsem E, Stein A, et al. Esmo consensus guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. Ann Oncol 2012;23(10):2479–516.

56. Kirstein MM, Lange A, Prenzler A, Manns MO, Kubicka S VA. Targeted Therapies in Metastatic Colorectal Cancer: A Systematic Review and Assessment of Currently Available Data. Oncologist 2014;19(11):1156–68.

57. De Gramont A, Van Cutsem E, Schmoll HJ, et al. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): A phase 3 randomised controlled trial. Lancet Oncol 2012;13(12):1225–33.

58. Fuchs CS, Marshall J, Mitchell E, et al. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: Results from the BICC-C study. J Clin Oncol 2007;25(30):4779–86.

59. Schmiegel W, Reinacher-Schick a, Arnold D, et al. Capecitabine/irinotecan or capecitabine/oxaliplatin in combination with bevacizumab is effective and safe as first-line therapy for metastatic colorectal cancer: a randomized phase II study of the AIO colorectal study group. Ann Oncol 2013;(3):1580–7.

60. Douillard JY, Siena S, Cassidy J, et al. Randomized, Phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) Versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: The PRIME study. J Clin Oncol 2010;28(31):4697–705.

61. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350(23):2335-42

62. Stintzing S, Modest DP, Rossius L, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): a post-hoc analysis of tumour dynamics in the final RAS wild-type subgroup of this randomised double-blind phase 3 trial. Lancet Oncol 2016;2045(16):1–9.

63. Elez E, Argilés G, Tabernero J. First-Line Treatment of Metastatic Colorectal Cancer: Interpreting FIRE-3, PEAK, and CALGB/SWOG 80405. Curr Treat Options Oncol 2015;16(11):52

64. Falcone A, Ricci S, Brunetti I, et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal

cancer: The gruppo oncologico nor. J Clin Oncol 2007;25(13):1670–6.

65. Loupakis F, Cremolini C, Masi G, et al. Initial Therapy with FOLFOXIRI and Bevacizumab for Metastatic Colorectal Cancer. N Engl J Med 2014;371(17):1609–18.

66. Gruenberger T, Bridgewater J, Chau I, et al. Bevacizumab plus mFOLFOX-6 or FOLFOXIRI in patients with initially unresectable liver metastases from colorectal cancer: The OLIVIA multinational randomised phase II trial. Ann Oncol 2015;26(4):702–8.

67. Souglakos J, Androulakis N, Syrigos K, et al. FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncolog. Br J Cancer 2006;94(6):798–805.

68. Tournigand C, André T, Achille E, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: A randomized GERCOR study. J Clin Oncol 2004;22(2):229–37.

69. Bennouna J, Sastre J, Arnold D, et al. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. Lancet Oncol 2013;14(1):29-37.

70. Giantonio BJ, Catalano PJ, Meropol NJ, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol 2007;25(12):1539-44.

71. Falcone A, Salvatore L, Boni L, et al. Continuation or reintroduction of bevacizumab beyond progression to first-line therapy in metastatic colorectal cancer: Final results of the randomized BEBYP trial. Ann Oncol 2015;26(4):724–30.

72. Van Cutsem E, Tabernero J, Lakomy R, et al. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. J Clin Oncol 2012;30(28):3499–506.

73. Tabernero J, Yoshino T, Cohn AL, et al. Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): A randomised, double-blin. Lancet Oncol 2015;16(5):499–508.

74. Sobrero AF, Maurel J, Fehrenbacher L, et al. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. J Clin Oncol 2008;26(14):2311-9

75. Van Cutsem E, Köhne C-H, Láng I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 2011;29(15):2011–9.

76. Hecht JR, Patnaik A, Berlin J, et al. Panitumumab monotherapy in patients with previously treated metastatic colorectal cancer. Cancer 2007;110(5):980–8.

77. Price TJ, Peeters M, Kim TW, et al. Panitumumab versus cetuximab in patients with chemotherapy-refractory wild-type KRAS exon 2 metastatic colorectal cancer (ASPECCT): A randomised, multicentre, open-label, non-inferiority phase 3 study. Lancet Oncol 2014;15(6):569–79.

78. Peeters M, Oliner KS, Parker A, et al. Massively parallel tumor multigene sequencing to

evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. Clin Cancer Res 2013;19(7):1902–12.

79. Grothey A, Van Cutsem E, Sobrero A, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet 2013;381(9863):303–12.

80. Li J, Qin S, Xu R, et al. Regorafenib plus best supportive care versus placebo plus best supportive care in Asian patients with previously treated metastatic colorectal cancer (CONCUR): A randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2015;16(6):619–29.

81. Mayer RJ, Van Cutsem E, Falcone A, et al. Randomized Trial of TAS-102 for Refractory Metastatic Colorectal Cancer. N Engl J Med 2015;372(20):1909–19.

82. Swain SM, Baselga J, Kim S-B, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med 2015;372(8):724–34.

83. Giuliano S, Pagès G. Mechanisms of resistance to anti-angiogenesis therapies. Biochimie. 2013;95(6):1110–9.

84. Lambrechts D, Lenz HJ, De Haas S, Carmeliet P, Scherer SJ. Markers of response for the antiangiogenic agent bevacizumab. J. Clin. Oncol. 2013;31(9):1219–30.

85. Douillard J-Y, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 2013;369(11):1023–34.

86. Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon R a, Karapetis CS. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized controlled trials. Ann Oncol 2015;26(1):13–21.

87. Tran B, Kopetz S, Tie J, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 2011;117(20):4623–32.

88. Seymour MT, Brown SR, Middleton G, et al. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): A prospectively stratified randomised trial. Lancet Oncol 2013;14(8):749–59.

89. Pietrantonio F, Petrelli F, Coinu A, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: A meta-analysis. Eur J Cancer 2015;51(5):587–94.

90. Rowland a, Dias MM, Wiese MD, et al. Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. Br J Cancer 2015;112(12):1888–94.

91. Cremolini C, Loupakis F, Antoniotti C, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: Updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. Lancet Oncol 2015;16(13):1306–15.

92. Hutchins G, Southward K, Handley K, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol 2011;29(10):1261–70.

93. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N

Engl J Med 2003;349(3):247–57.

94. Venderbosch S, Nagtegaal ID, Maughan TS, et al. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: A pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clin Cancer Res 2014;20(20):5322–30.

95. Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med 2015;372(26):2509–20.

96. Giampieri R, Scartozzi M, Del Prete M, et al. Molecular biomarkers of resistance to anti-EGFR treatment in metastatic colorectal cancer, from classical to innovation. Crit Rev Oncol Hematol 2013;88(2):272–83.

97. Moutinho C, Martinez-Cardús A, Santos C, et al. Epigenetic inactivation of the BRCA1 interactor SRBC and resistance to oxaliplatin in colorectal cancer. J Natl Cancer Inst 2014;106(1):1–9.

98. Bononi A, Agnoletto C, De Marchi E, et al. Protein kinases and phosphatases in the control of cell fate. Enzym Res 2011;2011:329098.

99. Perrotti D, Neviani P. Protein phosphatase 2A: a target for anticancer therapy. Lancet Oncol 2013;14(6):e229–38.

100. Trotman LC, Wang X, Alimonti A, et al. Ubiquitination Regulates PTEN Nuclear Import and Tumor Suppression. Cell 2007;128(1):141–56.

101. Eichhorn PJA, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. Biochim Biophys Acta 2009;1795(1):1–15.

102. Götz J, Probst A, Mistl C, Nitsch RM, Ehler E. Distinct role of protein phosphatase 2A subunit C*α* in the regulation of E-cadherin and *β*-catenin during development. Mech Dev 2000;93(1–2):83–93.

103. Janssens V, Longin S, Goris J. PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). Trends Biochem Sci 2008;33(3):113–21.

104. Chen J, Parsons S, Brautigan DL. Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. J Biol Chem 1994;269(11):7957–62.

105. Groves MR, Hanlon N, Turowski P, Hemmings BA, Barford D. The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. Cell 1999;96(1):99–110.

106. Xu Y, Xing Y, Chen Y, et al. Structure of the Protein Phosphatase 2A Holoenzyme. Cell 2006;127(6):1239–51.

107. Cho US, Xu W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. Nature 2007;445(7123):53–7.

108. Zhou J, Pham HT, Ruediger R, Walter G. Characterization of Aα and Aβ subunit isoforms of PP2A: differences in expression, subunit interaction, and evolution. Biochem J 2003;369(2):387–98.

109. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 2001;353(3):417–39.

110. Van Hoof C, Goris J. Phosphatases in apoptosis: To be or not to be, PP2A is in the heart of the question. Biochim Biophys Acta - Mol Cell Res 2003;1640(2–3):97–104.

111. Li X, Virshup DM. Two conserved domains in regulatory B subunits mediate binding to

the A subunit of protein phosphatase 2A. Eur J Biochem 2002;269(2):546–52.

112. Strack S, Chang D, Zaucha JA, Colbran RJ, Wadzinski BE. Cloning and characterization of Bδ, a novel regulatory subunit of protein phosphatase 2A. FEBS Lett 1999;460(3):462–6.

113. Strack S, Zaucha J a, Ebner FF, Colbran RJ, Wadzinski BE. Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. J Comp Neurol 1998;392(4):515–27.

114. McCright B, Virshup DM. Identification of a new family of protein phosphatase 2A regulatory subunits. J Biol Chem 1995;270(44):26123–8.

115. McCright B, Rivers AM, Audlin S et al. The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J Biol Chem 1996;271(36):22081–9.

116. Shouse GP, Nobumori Y, Panowicz MJ, Liu X. ATM-mediated phosphorylation activates the tumor-suppressive function of B56γ-PP2A. Oncogene 2011;30(35):3755–65.

117. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell 2005;8(5):355–68.

118. Böckelman C, Koskensalo S, Hagström J, Lundin M, Ristimäki A, Haglund C. CIP2A overexpression is associated with c-Myc expression in colorectal cancer. Cancer Biol Ther 2012;13(5):289–95.

119. Junttila MR, Puustinen P, Niemelä M, et al. CIP2A Inhibits PP2A in Human Malignancies. Cell 2007;130(1):51–62.

120. Mcconnell J, Gomez R, Mccorvey L, Law B, Wadzinski B. Identification of a PP2A-interacting protein that functions as a negative regulator of phosphatase activity in the ATM/ATR signaling pathway. Oncogene 2007;26(41):6021–30.

121. Kurimchak A, Graña X. PP2A holoenzymes negatively and positively regulate cell cycle progression by dephosphorylating pocket proteins and multiple CDK substrates. Gene 2012;499(1):1–7.

122. Ross JA, Cheng H, Nagy ZS, Frost JA, Kirken RA. Protein Phosphatase 2A Regulates Interleukin-2 Receptor Complex Formation and JAK3/STAT5 Activation. J Biol Chem 2010;285(6):3582–91.

123. Li Y, Yue P, Deng X, et al. Protein phosphatase 2A negatively regulates eukaryotic initiation factor 4E phosphorylation and eIF4F assembly through direct dephosphorylation of Mnk and eIF4E. Neoplasia 2010;12(10):848–55.

124. Pallas DC, Shahrik LK, Martin BL, et al. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell 1990;60(1):167–76.

125. Cristóbal I, Garcia-Orti L, Cirauqui C, et al. Overexpression of SET is a recurrent event associated with poor outcome and contributes to protein phosphatase 2A inhibition in acute myeloid leukemia. Haematologica 2012;97(4):543–50.

126. Sangodkar J, Farrington C, Mcclinch K etc al. All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. FEBS J 2017;283(6):1004–24.

127. Mannava S, Omilian A, Wawrzyniak J, et al. PP2A-B56α controls oncogene-induced senescence in normal and tumor human melanocytic cells. Oncogene 2012;31339(12):1484–92.

128. Ruvolo PP, Qui YH, Coombes KR, et al. Low expression of PP2A regulatory subunit B55α is associated with T308 phosphorylation of AKT and shorter complete remission duration in acute myeloid leukemia patients. Leukemia 2011;25(11):1711–7.

129. Curtis C, Shah SP, Chin S-F, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486(7403):346–52.

130. Chou HC, Chen CH, Lee HS, et al. Alterations of tumour suppressor gene PPP2R1B in hepatocellular carcinoma. Cancer Lett 2007;253(1):138–43.

131. Kalla C, Scheuermann MO, Kube I, et al. Analysis of 11q22-q23 deletion target genes in B-cell chronic lymphocytic leukaemia: Evidence for a pathogenic role of NPAT, CUL5, and PPP2R1B. Eur J Cancer 2007;43(8):1328–35.

132. Shih IM, Panuganti PK, Kuo KT, et al. Somatic mutations of PPP2R1A in ovarian and uterine carcinomas. Am J Pathol 2011;178(4):1442–7.

133. Cristóbal I, Garcia-Orti L, Cirauqui C, Alonso MM, Calasanz MJ, Odero MD. PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. Leuk Off J Leuk Soc Am Leuk Res Fund, UK 2011;25(4):606–14.

134. Yuan Q, Li PD, Li BH, et al. Differential IL-4/Stat6 activities correlate with differential expression of regulatory genes SOCS-1, SHP-1, and PP2A in colon cancer cells. J Cancer Res Clin Oncol 2009;135(1):131–40.

135. Bos CL, Kodach LL, van den Brink GR, et al. Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. Oncogene 2006;25(49):6447–56.

136. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, et al. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. J Biol Chem 2003;278(2):1158–64.

137. Adachi Y, Pavlakis GN, Copeland TD. Identification and characterization of SET, a nuclear phosphoprotein encoded by the translocation break point in acute undifferentiated leukemia. J Biol Chem 1994;269(3)2258–62.

138. Vlierberghe P Van, Grotel M Van, Tchinda J, et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. Pharmacia 2008;111(9):4668–80.

139. Chao A, Tsai CL, Wei PC, et al. Decreased expression of microRNA-199b increases protein levels of SET (protein phosphatase 2A inhibitor) in human choriocarcinoma. Cancer Lett 2010;291(1):99–107.

140. Carujo S, Estanyol JM, Ejarque a, Agell N, Bachs O, Pujol MJ. Glyceraldehyde 3-phosphate dehydrogenase is a SET-binding protein and regulates cyclin B-cdk1 activity. Oncogene 2006;25(29):4033–42.

141. Compagnone N a, Zhang P, Vigne JL, Mellon SH. Novel role for the nuclear phosphoprotein SET in transcriptional activation of P450c17 and initiation of neurosteroidogenesis. Mol Endocrinol 2000;14(6):875–88.

142. Ten Klooster JP, Leeuwen I V, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET. EMBO J 2007;26(2):336–45.

143. Cervoni N, Detich N, Seo SB, Chakravarti D, Szyf M. The oncoprotein set/TAF-1, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem 2002;277(28):25026–31.

144. Almeida LO, Garcia CB, Matos-Silva FA, Curti C, Leopoldino AM. Accumulated SET protein up-regulates and interacts with hnRNPK, increasing its binding to nucleic acids, the Bcl-xS repression, and cellular proliferation. Biochem Biophys Res Commun 2014;445(1):196–202.

145. Al-Murrani SW, Woodgett JR, Damuni Z. Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. Biochem J 1999;341(2):293–8.

146. Schmitz MHA, Held M, Janssens V, et al. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. Nat Cell Biol 2010;12(9):886–93.

147. Pippa R, Dominguez A, Malumbres R, et al. MYC-dependent recruitment of RUNX1 and GATA2 on the SET oncogene promoter enhances PP2A inactivation in acute myeloid leukemia. Oncotarget 2016;5(4):24–6.

148. Carlson SG, Eng E, Kim EG, Ballermann J. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor. J Am Soc Nephrol 1998;(10)1873–80.

149. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein set is its inhibitor. Cell 2003;112(5):659–72.

150. Westermarck J, Hahn WC. Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med 2008;14(4):152–60.

151. Fukukawa C, Shima H, Tanuma N. Up-regulation of I-2 PP2A / SET gene expression in rat primary hepatomas and regenerating livers. Cancer Lett 2000;161(1):89–95.

152. Leopoldino AM, Squarize CH, Garcia CB, et al. SET protein accumulates in HNSCC and contributes to cell survival: Antioxidant defense, Akt phosphorylation and AVOs acidification. Oral Oncol 2012;48(11):1106–13.

153. Janghorban M, Farrell AS, Allen-Petersen BL, et al. Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer. Proc Natl Acad Sci U S A 2014;111(25):9157–62.

154. Sobral LM, Sousa LO, Coletta RD, et al. Stable SET knockdown in head and neck squamous cell carcinoma promotes cell invasion and the mesenchymal-like phenotype in vitro, as well as necrosis, cisplatin sensitivity and lymph node metastasis in xenograft tumor models. Mol Cancer 2014;13:32.

155. Saddoughi SA, Gencer S, Peterson YK, et al. Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumour suppression via activation of PP2A-RIPK1-dependent necroptosis. EMBO Mol Med 2013;5(1):105–21.

156. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. MicroRNAs in cancer management. Lancet Oncol. 2012;13(6):e249-58

157. Xuan Y, Yang H, Zhao L, et al. MicroRNAs in colorectal cancer: Small molecules with big functions. Cancer Lett 2015;360(2):89–105.

158. Friedman RC, Farh KKH, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19(1):92–105.

159. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99(24):15524–9.

160. Cimmino A, Calin GA, Fabbri M, Lorio M V, Ferracin M, et al. miR-15 and miR-16 induce

apoptosis by targeting BCL2. Proc Natl Acad Sci USA 2005;102(39):13944-9

161. Cekaite L, Eide PW, Lind GE, Skotheim RI, Lothe RA. MicroRNAs as growth regulators, their function and biomarker status in colorectal cancer. Oncotarget 2016;7(6):6476–505.

162. O'Donnell K a, Wentzel E a, Zeller KI, Dang C V, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435(7043):839–43.

163. Chang T-C, Zeitels LR, Hwang H-W, et al. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc Natl Acad Sci U S A 2009;106(9):3384–9.

164. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer 2010;17(1).19-36

165. Watahiki A, Wang Y, Morris J, et al. MicroRNAs associated with metastatic prostate cancer. PLoS One 2011;6(9):e24950

166. Toyota M, Suzuki H, Sasaki Y, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 2008;68(11):4123–32.

167. Chin LJ, Ratner E, Leng S, et al. A SNP in a let-7 microRNA complementary site in the KRAS 3 untranslated region increases non-small cell lung cancer risk. Cancer Res 2008;68(20):8535–40.

168. Takebe N, Harris PJ, Warren RQ, Ivy SP. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nat Rev Clin Oncol 2011;8(2):97–106.

169. Hua Z, Lv Q, Ye W, et al. Mirna-directed regulation of VEGF and other angiogenic under hypoxia. PLoS One 2006;1(1):e116

170. Toiyama Y, Okugawa Y, Goel A. DNA methylation and microRNA biomarkers for noninvasive detection of gastric and colorectal cancer. Biochem Biophys Res Commun 2014;455(1–2):43–57.

171. Oberg AL, French AJ, Sarver AL, et al. MiRNA expression in colon polyps provides evidence for a Multihit model of colon cancer. PLoS One 2011;6(6): e20465

172. Arndt GM, Dossey L, Cullen LM, et al. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC Cancer 2009;9(1):1–17.

173. Xiong B, Cheng Y, Ma L, Zhang C. MiR-21 regulates biological behavior through the PTEN/PI-3 K/Akt signaling pathway in human colorectal cancer cells. Int J Oncol 2013;42(1):219–28.

174. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120(5):635–47.

175. Pagliuca a, Valvo C, Fabrizi E, et al. Analysis of the combined action of miR-143 and miR-145 on oncogenic pathways in colorectal cancer cells reveals a coordinate program of gene repression. Oncogene 2013;32(40):4806–13.

176. Gregory PA, Bracken CP, Smith E, et al. An autocrine TGF-beta/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. Mol Biol Cell 2011;22(10):1686–98.

177. Faber C, Kirchner T, Hlubek F. The impact of microRNAs on colorectal cancer. Virchows Arch 2009;454(4):359–67.

178. Ng EKO, Chong WWS, Jin H, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 2009;58(10):1375–81.

179. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 2010;127(1):118–26.

180. Schetter AJ, Leung S, Sohn JJ, et al. MIcrorna expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. Jama 2008;299(4):425–36.

181. Schee K, Lorenz S, Worren MM, et al. Deep Sequencing the MicroRNA Transcriptome in Colorectal Cancer. PLoS One 2013;8(6): e66165.

182. Toiyama Y, Takahashi M, Hur K, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst 2013;105(12):849–59.

183. Ogata-Kawata H, Izumiya M, Kurioka D, et al. Circulating exosomal microRNAs as biomarkers of colon cancer. PLoS One 2014;9(4):e92921

184. Lv Z chuan, Fan Y sheng, Chen H bing, Zhao D wei. Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer. Tumor Biol 2014;36(3):1619–25.

185. Pu XX, Huang GL, Guo HQ, et al. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. J Gastroenterol Hepatol 2010;25(10):1674–80.

186. Kanaan Z, Roberts H, Eichenberger MR, et al. A Plasma MicroRNA Panel for Detection of Colorectal Adenomas. Ann Surg 2013;258(3):400–8.

187. Wang S, Xiang J, Li Z, et al. A plasma microRNA panel for early detection of colorectal cancer. Int J Cancer 2015;136(1):152–61.

188. Bovell LC, Shanmugam C, Putcha BDK, et al. The prognostic value of MicroRNAs varies with patient race/ethnicity and stage of colorectal cancer. Clin Cancer Res 2013;19(14):3955–65.

189. Zhang J-X, Song W, Chen Z-H, et al. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. Lancet Oncol 2013;14(13):1295–306.

190. Nosho K, Igarashi H, Nojima M, et al. Association of microRNA-31 with BRAF mutation, colorectal cancer survival and serrated pathway. Carcinogenesis 2014;35(4):776–83.

191. Hur K, Toiyama Y, Schetter AJ, et al. Identification of a Metastasis-Specific MicroRNA Signature in Human Colorectal Cancer. Jcni 2015;107(3):1–11.

192. Schepeler T, Reinert JT, Ostenfeld MS, et al. Diagnostic and prognostic microRNAs in stage II colon cancer. Cancer Res 2008;68(15):6416–24.

193. Manceau G, Imbeaud S, Thiébaut R, et al. Hsa-miR-31-3p expression is linked to progression-free survival in patients with KRAS wild-type metastatic colorectal cancer treated with anti-EGFR therapy. Clin Cancer Res 2014;20(12):3338–47.

194. Favreau AJ, McGlauflin RE, Duarte CW, Sathyanarayana P. miR-199b, a novel tumor suppressor miRNA in acute myeloid leukemia with prognostic implications. Exp Hematol Oncol 2015;5:4.

195. Sousa LO, Sobral LM, Matsumoto CS, et al. Lymph node or perineural invasion is associated with low miR-15a, miR-34c and miR-199b levels in head and neck squamous

cell carcinoma. BBA Clin 2016;6:159–64.

196. Fang C, Wang F-B, Li Y, Zeng X-T. Down-regulation of miR-199b-5p is correlated with poor prognosis for breast cancer patients. Biomed Pharmacother 2016;84:1189–93.

197. Shang W, Chen X, Nie L, et al. MiR199b suppresses expression of hypoxia-inducible factor 1alpha (HIF-1alpha) in prostate cancer cells. Int J Mol Sci 2013;14(4):8422–36.

198. Zeng H, Zhang Z, Dai X, Chen Y, Ye J, Jin Z. Increased Expression of microRNA-199b-5p Associates with Poor Prognosis Through Promoting Cell Proliferation, Invasion and Migration Abilities of Human Osteosarcoma. Pathol Oncol Res 2016;22(2):253–60.

199. Andolfo I, Liguori L, Antonellis P De, et al. The micro-RNA 199b-5p regulatory circuit. 2012;14(5):596–612.

200. Joshi D, Chandrakala S, Korgaonkar S, Ghosh K, Vundinti BR. Down-regulation of miR-199b associated with imatinib drug resistance in 9q34.1 deleted BCR/ABL positive CML patients. Gene 2014;542(2):109–12.

201. Liu MX, Siu MKY, Liu SS, Yam JWP, Ngan HYS, Chan DW. Epigenetic silencing of microRNA-199b-5p is associated with acquired chemoresistance via activation of JAG1-Notch1 signaling in ovarian cancer. Oncotarget 2014;5(4):944–58.

202. Garzia L, Andolfo I, Cusanelli E, et al. MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. PLoS One 2009;4(3):e4998

203. Wang C, Song B, Song W, et al. Underexpressed microRNA-199b-5p targets Hypoxia-Inducible Factor-1alpha in hepatocellular carcinoma and predicts prognosis of hepatocellular carcinoma patients. J Gastroenterol Hepatol 2011;26(11):1630–7.

204. Fang C, Zhao Y, Guo B. MiR-199b-5p targets HER2 in breast cancer cells. J Cell Biochem 2013;114(7):1457–63.

205. Zhang Q, Padi SKR, Tindall DJ, Guo B. Polycomb protein EZH2 suppresses apoptosis by silencing the proapoptotic miR-31. Cell Death Dis 2014;5(10):e1486-7.

206. Valastyan S, Weinberg RA. miR-31: A crucial overseer of tumor metastasis and other emerging roles. Cell Cycle 2010;9(11):2124–9.

207. Stepicheva NA, Song JL. Function and regulation of microRNA-31 in development and disease. Mol Reprod Dev 2016;83(8):654–74.

208. Bandrés E, Cubedo E, Agirre X, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer 2006;5:29

209. Sun D, Yu F, Ma Y, et al. MicroRNA-31 activates the RAS pathway and functions as an oncogenic MicroRNA in human colorectal cancer by repressing RAS p21 GTPase activating protein 1 (RASA1). J Biol Chem 2013;288(13):9508–18.

210. Wang C-J, Stratmann J, Zhou Z-G, Sun X-F. Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. BMC Cancer 2010;10(1):616.

211. Mlcochova J, Faltejskova-Vychytilova P, Ferracin M, et al. MicroRNA expression profiling identifies miR-31-5p/3p as associated with time to progression in wild-type RAS metastatic colorectal cancer treated with cetuximab. Oncotarget 2015;6(36):38695–704.

212. Liu X, Sempere LF, Ouyang H, et al. MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. J Clin

Invest 2010;120(4):1298-309

213. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 2004;10(12):1957–66.

214. Pfeffer SR, Yang CH, Pfeffer LM. The Role of MIR-21 in Cancer. Drug Dev Res 2015;76(6):270–7.

215. Grady WM, Carethers JM. Genomic and Epigenetic Instability in Colorectal Cancer Pathogenesis. Gastroenterology 2008;135(4):1079–99.

216. Zhao J, Zhang Y, Zhao G. Emerging role of microRNA-21 in colorectal cancer. Cancer Biomarkers 2015;15(3):219–26.

217. Ma X, Buscaglia LEB, Barker JR, Li Y. MicroRNAs in NF- k B signaling. J Mol Cell Biol 2011;3(3):159–66.

218. Toiyama Y, Okugawa Y, Goel A. Dna Methylation and Microrna Biomarkers for Noninvasive Detection of Gastric and Colorectal Cancer. Biochem Biophys Res Commun 2014;455(1-2):43-57.

219. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat 2006;100(2):229–35.

220. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008;3(6):1101–8.

221. Van Cutsem E, Cervantes A, Nordlinger B, Arnold D, The ESMO Guidelines Working Group. Metastatic colorectal cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2014;25(3):iii1-9.

222. Ripoli FL, Mohr A, Hammer SC, et al. A comparison of fresh frozen vs. Formalin-fixed, paraffin-embedded specimens of canine mammary tumors via branched-DNA assay. Int J Mol Sci 2016;17(5).pii: E724.

223. Min H, Yoon S. Got target? Computational methods for microRNA target prediction and their extension.Exp Mol Med 2010;42(4):233–44.

224. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell 2009;136(2):215–33.

225. Kiely M, Kiely PA. PP2A: The wolf in sheeps clothing? Cancers 2015;7(2):648–69.

226. Takagi Y, Futamura M, Yamaguchi K, Aoki S, Takahashi T, Saji S. Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers. Gut 2000;47(2):268–71.

227. Ruediger R, Pham HT, Walter G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A a subunit gene. Oncogene 2001;20(1):10–5.

228. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science 1992;257(5074):1261–4.

229. Shen ZZ-LZ, Wang B, Jiang K-WKK-WK, et al. Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. Oncotarget 2016;7(23):35092–105.

230. Cheng Y, Liu W, Kim S, et al. Evaluation of PPP2R2A as a prostate cancer susceptibility gene: a comprehensive germline and somatic study. Cancer Genet 2011;204(7):375–81.

231. Grochola LF, Vazquez A, Bond EE, et al. Recent natural selection identifies a genetic

variant in a regulatory subunit of protein phosphatase 2A that associates with altered cancer risk and survival. Clin Cancer Res 2009;15(19):6301–8.

232. Muppala S, Mudduluru G, Leupold JH, Buergy D, Sleeman JP, Allgayer H. CD24 Induces Expression of the Oncomir miR-21 via Src, and CD24 and Src Are Both Post-Transcriptionally Downregulated by the Tumor Suppressor miR-34a. PLoS One 2013;8(3):e59563.

233. Eichhorn PJA, Creyghton MP, Wilhelmsen K, Van Dam H, Bernards R. A RNA interference screen identifies the protein phosphatase 2A subunit PR55γ as a stress-sensitive inhibitor of c-SRC. PLoS Genet 2007;3(12):2381–94.

234. Buscaglia LEB, Li Y. Apoptosis and the target genes of microRNA-21. Chin J Cancer 2011;30(6):371–80.

235. Casado E, Garcia VM, Sanchez JJ, et al. A combined strategy of SAGE and quantitative PCR provides a 13-gene signature that predicts preoperative chemoradiotherapy response and outcome in rectal cancer. Clin Cancer Res 2011;17(12):4145–54.

236. Hoshinob Y, Company T, Industries YP. CsA was initially isolated from Trichoderma polysporum5f6. Gazz Chim Ital 1994;5–6.

237. White C, Alshaker H, Cooper C, Winkler M, Pchejetski D. The emerging role of FTY720 (Fingolimod) in cancer treatment. Oncotarget 2016;7(17):23106–27.

238. Suzuki S, Li XK, Enosawa S, Shinomiya T. A new immunosuppressant, FTY720, induces bcl-2-associated apoptotic cell death in human lymphocytes. Immunology 1996;89(4):518–23.

239. Yoshino T, Tabunoki H, Sugiyama S, Ishii K, Kim SU, Satoh J. Non-phosphorylated FTY720 Induces Apoptosis of Human Microglia by Activating SREBP2. Cell Mol Neurobiol 2011;31(7):1009–20.

240. Zhou C, Ling MT, Kin-Wah Lee T, Man K, Wang X, Wong YC. FTY720, a fungus metabolite, inhibits invasion ability of androgen-independent prostate cancer cells through inactivation of RhoA-GTPase. Cancer Lett 2006;233(1):36–47.

241. Azuma H, Takahara S, Ichimaru N, et al. Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. Cancer Res 2002;62(5):1410–9.

242. Liu Q, Zhao X, Frissora F, et al. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia / lymphoma. 2013;111(1):275–84.

243. Salinas NRA, Oshima CTF, Cury PM, Cordeiro JA, Bueno V. FTY720 and lung tumor development. Int Immunopharmacol 2009;9(6):689–93.

244. Ho JWY, Man K, Sun CK, Lee TK, Poon RTP, Fan ST. Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma. Mol Cancer Ther 2005;4(9):1430–8.

245. Shen Y, Cai M, Xia W, et al. FTY720, a synthetic compound from Isaria sinclairii, inhibits proliferation and induces apoptosis in pancreatic cancer cells. Cancer Lett 2007;254(2):288–97.

246. Nagaoka Y, Otsuki K, Fujita T, Uesato S. Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast and colon cancer cells. Biol Pharm Bull 2008;31(6):1177–81.

247. Liu R, Tang J, Ding C, et al. The depletion of ATM inhibits colon cancer proliferation and migration via B56γ2-mediated Chk1/p53/CD44 cascades. Cancer Lett 2017;390(17):48–57.

248. Di Conza G, Trusso Cafarello S, Loroch S, et al. The mTOR and PP2A Pathways Regulate PHD2 Phosphorylation to Fine-Tune HIF1α Levels and Colorectal Cancer Cell Survival under Hypoxia. Cell Rep 2017;18(7):1699–712.

249. Liu C-C, Lin S-P, Hsu H-S, et al. Suspension survival mediated by PP2A-STAT3-Col XVII determines tumour initiation and metastasis in cancer stem cells. Nat Commun 2016;7:14027.

250. Lopes-Ramos C, Habr-Gama A, Quevedo B, et al. Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients. BMC Med Genomics 2014;7(1):68.